

Oxidativer Stress im Alter und bei der Alzheimer Krankheit

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Eine vergleichende Studie über oxidative Schäden
und antioxidative Enzymaktivitäten
in Mausmodellen und humanem Gehirngewebe

Dissertation
zur Erlangung des Doktorgrades der Naturwissenschaften
(Dr. phil. nat.)

vorgelegt beim Fachbereich Chemische und
Pharmazeutische Wissenschaften
der Johann Wolfgang Goethe - Universität
in Frankfurt am Main

von
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Frankfurt (2004)
(D F 1)

vom Fachbereich Chemische und Pharmazeutische Wissenschaften der Johann
Wolfgang Goethe – Universität als Dissertation angenommen.

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Datum der Disputation: 20. 12. 2004

OXIDATIVE STRESS DURING AGING AND IN ALZHEIMER'S DISEASE

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A COMPARATIVE STUDY OF OXIDATIVE DAMAGE AND
ANTIOXIDANT ENZYMATIC ACTIVITIES
IN MOUSE MODELS AND HUMAN BRAIN TISSUE



THESIS
FOR THE DEGREE OF
DOCTOR OF NATURAL SCIENCES
(DR. PHIL. NAT.)

SUBMITTED TO THE FACULTY OF CHEMICAL AND
PHARMACEUTICAL SCIENCES OF THE
JOHANN WOLFGANG GOETHE - UNIVERSITY
FRANKFURT / MAIN

BY
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FROM PASSAU

FRANKFURT (2004)

Der Glaube versetzt Berge, der Zweifel erklettert sie.

Karl Heinrich Waggerl

Meiner Familie und Matthias gewidmet.

TABLE OF CONTENTS

1	INTRODUCTION	1
1.1	OXYGEN AND OXIDATIVE STRESS.....	2
1.1.1	<i>Formation of reactive oxygen species</i>	2
1.1.2	<i>Physiological roles of reactive oxygen species.....</i>	4
1.1.3	<i>Oxidative stress.....</i>	5
1.1.3.1	Increased production of ROS from endogenous sources.....	5
1.1.3.2	Antioxidant defense	7
1.1.3.3	Oxidative attack on cellular molecules	10
1.1.3.4	Intracellular signaling and transcription factors activated by ROS	13
1.1.3.5	Role of ROS in cell death	15
1.2	OXIDATIVE STRESS IN BRAIN AGING.....	18
1.2.1	<i>Aging theories.....</i>	18
1.2.2	<i>The free radical theory of aging</i>	19
1.2.3	<i>A role for oxidative stress in brain aging?</i>	20
1.3	OXIDATIVE STRESS AND ALZHEIMER'S DISEASE	23
1.3.1	<i>Alzheimer's disease – clinical symptoms, pathology and risk factors.....</i>	23
1.3.1.1	Clinical picture of Alzheimer's disease	23
1.3.1.2	AD neuropathology.....	24
1.3.1.3	Genetic and non-genetic risk factors for AD	26
1.3.2	<i>Risk factors for sporadic AD</i>	27
1.3.3	<i>Familial AD mutations and the amyloid beta hypothesis.....</i>	29
1.3.3.1	The amyloid precursor protein APP – physiological processing and effect of APP mutations	29
1.3.3.2	Presenilins.....	35
1.3.4	<i>A role for oxidative stress in AD?.....</i>	39
1.3.4.1	Oxidative stress in sporadic AD patients.....	39
1.3.4.2	Oxidative stress and A β toxicity.....	40
1.3.4.3	Oxidative stress and presenilins.....	41
1.4	AIMS OF THESIS.....	42
2	MATERIALS AND METHODS	45
2.1	MATERIALS.....	46
2.1.1	<i>Chemicals.....</i>	46

TABLE OF CONTENTS

2.1.2 Kits	48
2.1.3 Antibodies.....	48
2.1.4 Buffers and media	48
2.1.4.1 Buffers used for brain tissue preparation and homogenization.....	49
2.1.4.2 Buffers used for lymphocyte preparation.....	49
2.1.4.3 Buffers for Western blots	49
2.1.4.4 Buffers for DNA gel electrophoresis	50
2.1.5 Apparatus and other materials.....	50
2.1.6 Computer software	52
2.1.7 Mice.....	52
2.1.7.1 C57BL/6J mice.....	53
2.1.7.2 Transgenic mice	53
2.1.7.3 Summary of mice used in this thesis.....	55
2.1.8 Human brain tissue	56
2.1.8.1 Cohort #1.....	56
2.1.8.2 Cohort #2.....	57
2.2 METHODS	58
2.2.1 Preparation of tissues and cells	58
2.2.1.1 Preparation of murine brain tissue	58
2.2.1.2 Homogenization of human and murine brain tissue for determination of antioxidant enzyme activities and lipid peroxidation products.....	59
2.2.1.3 Isolation of murine splenic lymphocytes	59
2.2.2 Assays of antioxidant enzymes	59
2.2.2.1 Superoxide dismutase assay	59
2.2.2.2 Glutathione peroxidase assay	60
2.2.2.3 Glutathione reductase assay	61
2.2.3 Lipid peroxidation measurement.....	62
2.2.4 Determination of reactive oxygen species in isolated splenic lymphocytes...	63
2.2.4.1 Flow cytometric analysis of splenic lymphocytes.....	63
2.2.4.2 Staining of lymphocytes with ROS-sensitive fluorescent dyes	67
2.2.4.3 FACS analysis of splenic lymphocytes.....	70
2.2.5 Western Blot analysis of APP and A β	71
2.2.5.1 Preparation of brain samples for determination of soluble A β	71
2.2.5.2 Preparation of brain samples for determination of insoluble A β	72

2.2.5.3 SDS PAGE and Western Blotting of brain extracts	72
2.2.5.4 Detection of APP, C99 and Abeta	73
2.2.5.5 Detection of actin as loading control	73
2.2.6 Quantification of $A\beta_{1-40}$ by ELISA	73
2.2.7 Genotyping of transgenic mice	74
2.2.7.1 DNA isolation from rodent tails	74
2.2.7.2 PCR reaction	75
2.2.7.3 DNA gel electrophoresis.....	75
2.2.8 RT-PCR analysis of APP and PS1 expression in splenic lymphocytes.....	76
2.2.9 Determination of protein content.....	77
2.2.10 Calculations and Statistics.....	78
3 RESULTS	81
3.1 EFFECTS OF AGING AND GENDER ON ROS METABOLISM IN BRAIN TISSUE AND PERIPHERAL CELLS OF MICE	82
3.1.1 ROS metabolism in C57BL/6J mice during aging.....	82
3.1.1.1 Oxidative damage and enzymatic antioxidant defense in murine brains during aging	82
3.1.1.2 ROS levels in splenic lymphocytes during aging	86
3.1.2 Gender differences in ROS metabolism in C57BL/6J mice during aging	89
3.1.2.1 Gender differences in oxidative damage and enzymatic antioxidant defense in murine brains	89
3.1.2.2 Gender differences in ROS levels in splenic lymphocytes.....	92
3.2 ROS METABOLISM IN TRANSGENIC AD MOUSE MODELS	94
3.2.1 ROS metabolism in PDGF-APP and/or PS1 transgenic mice.....	95
3.2.1.1 Oxidative damage and enzymatic antioxidant defense in transgenic mouse brains	96
3.2.1.2 Analysis of splenic lymphocytes from PS1 transgenic mice.....	98
3.2.2 ROS metabolism in brains from Thy1-APP transgenic mice.....	107
3.2.2.1 ROS metabolism in brains from Thy1-APP transgenic mice during aging	108
3.2.2.2 Gender differences in ROS metabolism in Thy1-APP transgenic mice.....	111
3.2.3 Cu/Zn-SOD activity in brains from APP23 mice.....	116
3.2.4 Comparative analysis of human APP expression and $A\beta$ expression levels in PDGF-APP, PDGF-APP/PS1 and Thy1-APP mouse models.....	117

TABLE OF CONTENTS

3.2.4.1 Western blot analysis of human APP and A β expression.....	117
3.2.4.2 Quantitative analysis of soluble A β ₁₋₄₀ levels in APP transgenic mice with ELISA	122
3.3 ROS METABOLISM IN BRAINS FROM AD PATIENTS	126
3.3.1 Cohort #1	126
3.3.1.1 Increased antioxidant metabolism in AD patients.....	126
3.3.1.2 Effect of gender on antioxidant metabolism in AD patients	131
3.3.2 Cohort #2	133
3.3.2.1 Changes in oxidative stress parameters in AD patients	133
3.3.2.2 Levels of soluble A β ₁₋₄₀ in brains from AD patients – correlation with Apo E4 genotype	137
3.3.2.3 Correlations of lipid peroxidation products and antioxidant enzyme activities with levels of soluble A β ₁₋₄₀	140
3.3.2.4 Correlations of lipid peroxidation products and antioxidant enzyme activities with Apo E genotype	141
3.3.2.5 Correlations of lipid peroxidation products and antioxidant enzyme activities with mini mental status (MMSE) score	143
4 DISCUSSION	147
4.1 EFFECTS OF AGING AND GENDER ON ROS METABOLISM IN C57BL/6J MICE	148
4.1.1 Effect of aging on oxidative stress parameters in brain tissue	148
4.1.2 Effect of aging on ROS levels in peripheral cells.....	158
4.1.3 Gender differences in oxidative stress-related parameters in mice.....	163
4.1.4 Summary of aging-induced effects on ROS and oxidative stress parameters in C57BL/6J mice.....	168
4.2 OXIDATIVE STRESS-RELATED PARAMETERS IN TRANSGENIC MICE BEARING FAD MUTATIONS	172
4.2.1 Effect of PS1 mutations on oxidative stress-parameters in transgenic mice.....	172
4.2.2 Effects of APP mutations on oxidative stress-related parameters	186
4.2.2.1 Analysis of PDGF-APP and PDGF-APP/PS1 double transgenic mice	186
4.2.2.2 Analysis of Thy1-APP transgenic mice	188
4.2.2.3 Reduced activity of Cu/Zn-SOD in APP23 transgenic mice	196
4.2.3 Comparison of different AD transgenic mouse models.....	198

4.3 CHANGES IN OXIDATIVE STRESS-RELATED PARAMETERS IN SPORADIC AD PATIENTS	204
4.3.1 Cohort #1	204
4.3.1.1 Upregulation of antioxidant defense prevents oxidative damage	205
4.3.1.2 Gender differences in antioxidant metabolism and oxidative damage in AD patients	209
4.3.1.3 Summary of observations in cohort #1	211
4.3.2 Cohort #2	211
4.3.2.1 Increased oxidative damage despite partial upregulation of antioxidant defense	211
4.3.2.2 Correlation of oxidative stress-related parameters with levels of amyloid beta, Apo E genotype and clinical severity of dementia	212
4.3.2.3 Summary of observations in cohort #2	218
4.3.3 Comparison of results from cohort #1 and #2	218
4.4 COMPARATIVE SUMMARY AND PERSPECTIVES	226
4.4.1 Suitability of transgenic mice to study AD-relevant pathogenic mechanisms	226
4.4.2 Is HNE a major toxic factor in AD?	229
4.4.3 Gender differences in AD and hormone replacement therapy	231
4.4.4 Is it possible to prevent AD by antioxidants?	233
5 SUMMARY / ZUSAMMENFASSUNG.....	235
5.1 SUMMARY.....	236
5.1.1 The influence of aging studied in mice	236
5.1.2 Effects of FAD mutations on oxidative stress parameters	237
5.1.3 Studies on human brain tissue from sporadic AD patients.....	238
5.1.4 Comparative summary and conclusions	240
5.2 ZUSAMMENFASSUNG	242
5.2.1 Alterseffekte in Mäusen.....	242
5.2.2 Einfluß von familiären Alzheimer-Mutationen	243
5.2.3 Untersuchungen an humanem Gehirngewebe von sporadischen Alzheimerpatienten.....	245
5.2.4 Vergleichende Zusammenfassung und Fazit.....	247
6 REFERENCES.....	249

TABLE OF CONTENTS

7	ABBREVIATIONS	297
8	INDEX OF FIGURES AND TABLES	301
8.1	INDEX OF FIGURES	302
8.2	INDEX OF TABLES	304
9	BIBLIOGRAPHY	307
9.1	ORIGINAL PUBLICATIONS AND REVIEWS	308
9.2	POSTERS	308
9.3	ORAL PRESENTATIONS.....	309
10	ACKNOWLEDGMENTS / DANKSAGUNG	311
11	CURRICULUM VITAE	315

1 INTRODUCTION

1.1 Oxygen and oxidative stress

Considering the role of oxygen for life on our planet, it is a Janus-faced molecule. It is indispensable to aerobic bacteria, fungi and higher organisms for metabolism, while at the same time its metabolites can exhibit deleterious effects towards almost any kind of biological molecule. When oxygen first appeared in significant amounts in the atmosphere about 2.5 to 3 billion years ago, it set off an ecological catastrophe. Blue-green algae had acquired the ability to split water and release molecular oxygen by photosynthesis. Other primitive organisms at that time were essentially anaerobes, and the rising amounts of oxygen in the atmosphere put severe stress on them. Many organisms of that time must have died out, while others receded to environments into which oxygen did not penetrate and survived as anaerobic organisms. But rising oxygen levels also started an evolutionary process of adaptation by development of antioxidant defense systems. Organisms that acquired defenses against oxidative toxicity were able to evolve new metabolic pathways and moreover use oxygen for energy production. The evolution of oxidative phosphorylation in mitochondria for ATP production provided a highly efficient way to produce energy and thus boosted the emergence of multicellular organisms that have formed the unimaginable diversity and variety of living beings on our planet today (Ohno, 1997).

1.1.1 Formation of reactive oxygen species

Reactive oxygen species (ROS) are either free radicals or non-radical molecules. For estimation of potentially damaging effects, the reactivity of each individual ROS as well as its ability to cross biological membranes has to be considered.

Molecular oxygen O_2 contains two unpaired electrons in its outer orbital and is a biradical. This triplet oxygen is by itself relatively unreactive towards electron pair donors. However, it can be activated, e.g. by light, to singlet oxygen with antiparallel electrons and a free π orbital that readily accepts paired electrons.

Apart from these molecular forms of oxygen, radicals and other non-radical derivatives of oxygen can be formed in aerobic organisms. These are collectively referred to as reactive oxygen species ROS in this thesis. Although several ROS contain nitrogen and are therefore sometimes referred to as reactive nitrogen species, the oxygen in these molecules is the driving force for reactivity. Therefore, the term ROS is used for any of these species in this thesis. Important ROS and their main sources in living organisms

are given in Table 1.1.

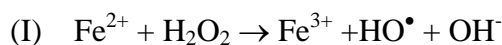
Table 1.1: Important ROS, their chemical formula and major sources.

nomenclature	formula	sources
superoxide	$O_2^{\bullet -}$	mitochondria, xanthine oxidase, nitric oxide synthase, NAD(P)H oxidase, oxidation of dopamine, adrenaline and noradrenaline
hydrogen peroxide	H_2O_2	superoxide dismutase, several oxidases
hydroperoxides	$ROOH$	cyclooxygenase, lipoxygenase, lipid peroxidation reactions
hydroxyl radical	HO^{\bullet}	Fenton reaction, hypochlorous acid, UV-induced fission of hydrogen peroxide
nitric oxide	NO^{\bullet}	nitric oxide synthases
peroxynitrite	$ONOO^-$	reaction of superoxide with nitric oxide
hypochlorous acid	$HOCl$	myeloperoxidase

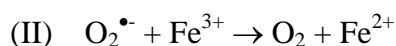
In a first step of electron transfer to molecular oxygen, superoxide radical anions are formed. The main source for this radical under physiological conditions is the respiratory chain in mitochondria. During the flow of electrons along the respiratory chain, leakage of electrons can occur. Electrons are then transferred directly to molecular oxygen under formation of the superoxide radical anion $O_2^{\bullet -}$. It is estimated that about 1-3 % of oxygen consumed in mitochondria are transformed to superoxide even under physiological conditions (Boveris and Chance, 1973). The exact step in the respiratory chain where electron leakage occurs is still under debate, but constituents of complex I (NADH dehydrogenase), semiquinones in complex II and cytochromes b from complex III have all been implicated. Leakage from complex IV (cytochrome c oxidase) is considered negligible (Turrens, 1997). Other sources of superoxide radicals include nitric oxide synthase enzymes (Xia et al., 1996; Xia and Zweier, 1997; Xia et al., 1998), and high levels of superoxide are also produced by NADPH oxidases in activated phagocytic cells (Rossi, 1986; Rosen et al., 1995). Superoxide radicals are highly reactive, but their damaging effect in cells is limited since diffusion across biological membranes is minimal due to the negative charge. However, other ROS can be derived from superoxide reactions, among these are hydrogen peroxide derived from further reduction as well as peroxynitrite formed in a reaction of superoxide with nitric oxide. Hydrogen peroxide has been detected in concentrations up to 100 μM in extracellular fluids under experimental conditions (Hyslop et al., 1995). Hydrogen

INTRODUCTION

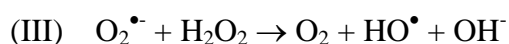
peroxide shows limited reactivity but can readily diffuse across membranes. It can be activated by transition metal ions like ferrous iron to form the highly reactive hydroxyl radical in the Fenton reaction (I):



Superoxide radicals can participate in Fenton reactions by reducing Fe^{3+} to Fe^{2+} and can thus propagate reaction (I) by providing reduced transition metal ions (II):



The net reaction of (I) and (II) is the so-called Haber-Weiss reaction (III):



Apart from iron, other transition metal ions, especially copper ions, can participate in the above reactions.

Nitrogen-containing ROS are derived from nitric oxide. Physiological levels of nitric oxide (NO) are estimated to range between 0.1 and 100 nM (Brown and Cooper, 1994). NO is to the largest part synthesized by nitric oxide synthases NOS. At least 3 different types of NOS have been identified, of which the endothelial eNOS and neuronal nNOS isoforms can be activated by increased intracellular calcium levels, whereas inducible iNOS is a calcium-independent enzyme (Bredt, 1999). iNOS activity leads to formation of higher NO levels than the other isoforms. Recently, a mitochondrial isoform of NOS has been identified, which is probably a splice variant of nNOS (Kanai et al., 2001). The main action of NO is the activation of soluble guanylate cyclase. NO can furthermore act as an antioxidant (Kanner et al., 1991), but can also form S-nitrosothiols with protein thiol groups thereby modulating or impairing protein function. NO can moreover inhibit complex IV of the mitochondrial respiratory chain or interact with superoxide anions to form peroxynitrite which exerts several toxic functions (Radi et al., 2002). Peroxynitrite can inhibit all mitochondrial respiratory chain complexes, initiate lipid peroxidation reactions and cause apoptotic cell death signaling (Brown and Borutaite, 2002).

1.1.2 Physiological roles of reactive oxygen species

Reactive oxygen species are not only by-products of aerobic respiration in mitochondria. ROS take part in a huge variety of enzymatic oxidation and reduction reactions involved in metabolism, cell signaling and neurotransmission. As an example,

the reactions of lipoxygenases and cyclooxygenases are dependent on as well as generate ROS. But ROS can also directly exert physiological functions, most importantly in immune functions, where phagocytic cells can form large amounts of superoxide anions and hypochlorous acid, which aid in the elimination of bacterial infections (Knight, 2000).

Also nitric oxide exerts several important physiological functions. Since its identification as vasorelaxant factor derived from endothelial cells (Ignarro et al., 1987), it has been detected in other cells and several physiological functions are still under investigation. Among these, NO seems to play important roles in immune function in the periphery, but also as a neurotransmitter and/or neuromodulator in the central nervous system (Hawkins et al., 1998).

1.1.3 Oxidative stress

In general, oxidative stress describes a state of imbalance between the production and detoxification of reactive oxygen species (Sies, 1991). Therefore, increased production of ROS as well as impaired antioxidant defense can both contribute to increased accumulation of ROS. ROS can be detrimental to almost any component of cells, including DNA, proteins and lipids. Cells react upon oxidative stress either by adaptive responses leading to activation of repair mechanisms or – if the damage is severe – by induction of cell death responses.

1.1.3.1 Increased production of ROS from endogenous sources

Under physiological conditions, ROS are mainly produced in mitochondria due to respiration. As stated in 1.1.1 (page 3), some leakage of electrons occurs along the respiratory chain resulting in production of superoxide radical anions. However, respiratory chain complexes are arranged in a manner that keeps leakage at low levels resulting in only 1-3 % production of superoxide relative to total oxygen consumption. If mitochondria are damaged by pathological conditions, superoxide production can be exacerbated due to disarrangement of mitochondrial respiratory chain complexes which results in higher electron leakage (Lenaz, 2001).

Another mechanism for ROS formation is an increased intracellular level of calcium. Endothelial and neuronal nitric oxide synthases eNOS and nNOS are activated by high intracellular calcium concentrations resulting in increased NO production (Bredt, 1999). In combination with superoxide anions, nitric oxide can react to form peroxynitrite.

INTRODUCTION

Furthermore, calcium activates phospholipase A₂ to release arachidonic acid from membrane phospholipids for synthesis of prostaglandins and leukotrienes by cyclooxygenase and lipoxygenase reactions, respectively. These reactions are accompanied by formation of lipid hydroperoxides (Kanner et al., 1987). Calcium also activates conversion of xanthine dehydrogenase to xanthine oxidase resulting in increased superoxide production (McCord, 1985). Finally, an important mechanism for calcium-induced ROS formation lies in mitochondrial calcium buffering. High intracellular calcium levels can be buffered by calcium uptake into mitochondria. High calcium levels however facilitate opening of the mitochondrial permeability transition pore resulting in mitochondrial swelling and release of mitochondrial factors into the cytosol, which is followed by a large increase in ROS formation and apoptotic signaling (Rego and Oliveira, 2003) (see 1.1.3.5, page 15 et seqq.).

Increased production of ROS has also been associated with ischemia/reperfusion injury and inflammatory conditions (McCord, 1985; Guzik et al., 2003; Ferrari et al., 2004). During ischemia, oxygen deprivation leads to a drop in ATP levels as well as collapse of the mitochondrial membrane potential and cellular damage. Once perfusion is restored, oxygen is provided to dysfunctional mitochondria which results in high ROS leakage from the inhibited respiratory chain [reviewed in (Halestrap et al., 2004)]. High levels of ROS are also produced from several cell types of the immune system, especially upon inflammatory activation. Inflammatory signals lead to activation of lipoxygenases and cyclooxygenases. Activated phagocytes and microglia in the brain can synthesize large amounts of superoxide radicals from NADPH oxidase reactions (Sankarapandi et al., 1998). Furthermore, hypochlorous acid is synthesized by myeloperoxidase reaction in neutrophils (Winterbourn, 2002). Therefore, inflammation is almost inevitably associated with oxidative stress.

As stated in 1.1.1 (page 4), free transition metal ions can favour Fenton chemistry and lead to formation of free radicals. Hence free metal ions – often derived from decomposition of metal-containing proteins or from exogenous sources – lead to increased ROS formation or formation of more reactive ROS. The toxicity of excess free metal ions is evident for example in haemochromatosis, where iron uptake is increased and exceeds the binding capacity of transferrin resulting in liver fibrosis, cardiac problems, arthritis and other symptoms, or in Wilson's disease, where copper overload causes liver dysfunction, metabolic deficiencies and mental retardation. Both disease states have been associated with increased markers of oxidative stress

(Brittenham et al., 2000; Hussain et al., 2000).

1.1.3.2 Antioxidant defense

Since ROS are produced even under physiological conditions, organisms need antioxidant defenses for their detoxification. Among these are low-molecular-mass antioxidants as well as antioxidant enzymes that often act in a complex interplay.

1.1.3.2.1 Low-molecular-mass antioxidants

Low-molecular-mass antioxidants are either produced endogenously, for example glutathione, uric acid, coenzyme Q, lipoic acid and bilirubin, or they are taken up by the diet. The most important endogenous antioxidant is the tripeptide glutathione. Glutathione is composed of the amino acids γ -glutamate, cysteine and glycine. The antioxidant properties of glutathione are due to the thiol residue in cysteine. Glutathione is found in millimolar concentrations in most mammalian cells (Cooper and Kristal, 1997). It can react either directly with ROS like superoxide, nitric oxide or the hydroxyl radical (Winterbourn and Metodiewa, 1994; Clancy et al., 1994) or act as cofactor for the enzymatic antioxidant defense by glutathione peroxidases. In brain, glutathione metabolism seems to be a complex interplay between astrocytes and neurons, where astrocytes have an essential function in providing neurons with glutathione precursors (Dringen, 2000).

The most prominent dietary antioxidants are vitamin C (ascorbic acid) and vitamin E (tocopherols). Vitamin C can donate one electron to free radicals under formation of the ascorbyl radical. Due to mesomeric stabilization of the free electron in the ascorbyl radical, this radical is very stable. Hence, vitamin C has the ability to intercept highly reactive free radicals. As a water-soluble antioxidant it can however not directly inactivate radicals in lipid bilayers of cell membranes, which are especially prone to free radical attack. This is afforded by tocopherols and tocotrienols that constitute the vitamin E family of molecules. Tocopherols and tocotrienols are lipid-soluble molecules that insert into cell membranes. They can donate a single electron to lipid peroxy radicals and thus effectively inhibit radical chain reactions. In addition, tocopherols can quench singlet oxygen. Notably, vitamin C acts to regenerate oxidized vitamin E (Packer et al., 1979; Leung et al., 1981).

1.1.3.2.2 Antioxidant enzymes

Aerobic organisms possess a variety of antioxidant enzymes that mostly act in concert to detoxify ROS (see Figure 1.1).

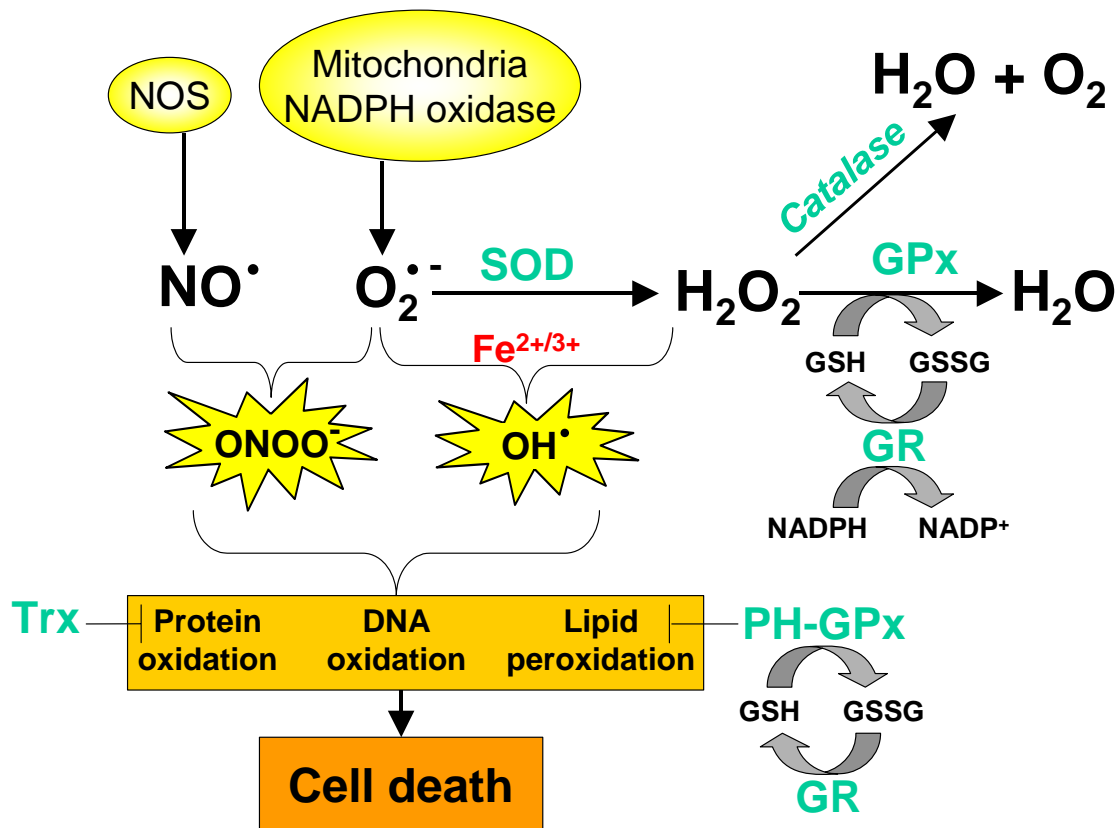
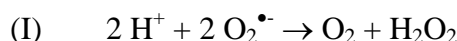


Figure 1.1: Pathways of formation and enzymatic detoxification of ROS.

GPx = glutathione peroxidase, GR = glutathione reductase, GSH = reduced glutathione, NOS = nitric oxide synthases, PH-GPx = phospholipidhydroperoxide glutathione peroxidase, SOD = superoxide dismutases, Trx = Thioredoxin.

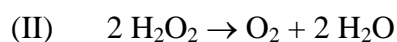
Superoxide radicals are derived from a first step of electron donation to molecular oxygen, and most other ROS can arise directly or indirectly from superoxide. Therefore superoxide-detoxifying enzymes act in the first line of enzymatic ROS defenses. Superoxide itself can undergo a dismutation reaction where two molecules of superoxide react to form molecular oxygen and hydrogen peroxide. This reaction is accelerated by superoxide dismutase SOD enzymes (I).



In eukaryotes, two different forms of superoxide dismutases have been identified: copper-zinc-dependent Cu/Zn-SOD (also termed SOD-1) and manganese-dependent Mn-SOD (SOD-2). While Mn-SOD is mainly localized to mitochondria, Cu/Zn-SOD has been found in high levels in cytosol but also in the intermembrane space between

the inner and outer mitochondrial membrane (Okado-Matsumoto and Fridovich, 2001). Furthermore, an extracellular form of Cu/Zn-SOD has also been identified (Fridovich, 1989). Since superoxide radicals are produced in mitochondria, mitochondrial Mn-SOD and Cu/Zn-SOD in the intermembrane space have important functions in preventing superoxide from entering the cytosol. Catalytic activity of Cu/Zn-SOD is dependent on copper ions, whereas zinc ions only help to stabilize protein structure (Fridovich, 1989).

Superoxide dismutase reactions result in formation of hydrogen peroxide which has to be decomposed further. This can be achieved by the reactions of catalase or glutathione peroxidases. While catalase activity directly inactivates hydrogen peroxide yielding water and molecular oxygen (II), glutathione peroxidases need reduced glutathione GSH as cosubstrate (III).

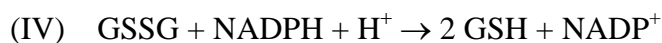


In view of the high K_m of catalase, glutathione peroxidases seem to be in large part responsible for removal of physiological hydrogen peroxide levels, whereas catalase activity becomes important at high hydrogen peroxide concentrations (Makino et al., 1994). Of note, catalase activity is low in brain (Marklund et al., 1982).

Four different types of glutathione peroxidases have been identified: (i) the “classical” glutathione peroxidase GPx, which is found intracellularly, (ii) phospholipid hydroperoxide glutathione peroxidase PH-GPx, which is located at the plasma membrane and can directly reduce lipid peroxidation products to the native lipids, (iii) an extracellular form of glutathione peroxidase as well as (iv) intestinal glutathione peroxidase in gastrointestinal tract and liver (Brigelius-Flohe, 1999). All of them are selenium-containing proteins. Classical glutathione peroxidase can convert hydrogen peroxide as well as simple organic hydroperoxides. Additionally, it can efficiently scavenge peroxynitrite (Sies and Arteel, 2000). Its subcellular localization is in the cytosol as well as mitochondrial matrix (Vitorica et al., 1984), where it may act in concert with SODs to decompose mitochondria-derived ROS. In brain tissue, GPx activity was mainly localized to astroglia (Damier et al., 1993; Takizawa et al., 1994), where also a high content of reduced glutathione was observed (Raps et al., 1989), indicating a prominent role for this antioxidant pathway in astroglia. GPx deficiency in mice sensitizes to oxidants (de Haan et al., 1998; Klivenyi et al., 2000) and ischemia (Crack et al., 2001). PH-GPx can also react with phospholipid hydroperoxides, which

makes it an especially important enzyme for inhibition of lipid peroxidation chain reactions. PH-GPx seems to play a very important physiological role since knockout of the gene proved to be embryonic lethal (Imai et al., 2003).

The reduced glutathione consumed by GPx reactions is restored from oxidized glutathione GSSG by the glutathione reductase GR reaction (IV).



The cofactor NADPH in this reaction is predominantly generated via the pentose phosphate pathway (Salvemini et al., 1999).

Further important enzymatic systems contributing to antioxidant defense include the thioredoxin/thioredoxin reductase system as well as glutathione S-transferases. Thioredoxin is a polypeptide containing two adjacent thiol groups that can undergo redox reactions with multiple proteins. Reduced thioredoxin is regenerated by thioredoxin reductases (Chae et al., 1999). Hence the main function of thioredoxin is the regeneration of proteins by reduction of disulfide bonds formed under oxidative conditions.

Glutathione S-transferases catalyse the formation of glutathione conjugates, which is especially important in the metabolism of xenobiotics. They also react with organic peroxides to form GSSG and the respective alcohols, which is essentially a glutathione peroxidase-like reaction. Glutathione S-transferases are also involved in detoxification of 4-hydroxynonenal (Goon et al., 1993; Hartley et al., 1995), a cytotoxic aldehyde derived from lipid peroxidation reactions (see 1.1.3.3, page 13).

1.1.3.3 Oxidative attack on cellular molecules

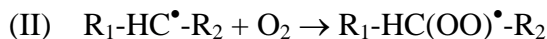
If ROS are not intercepted by antioxidant enzymes, they can lead to oxidative modification of essentially any cellular macromolecule or low-molecular weight component. The resulting products usually lose their function or even exhibit further damaging effects.

Lipid peroxidation

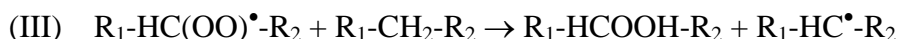
Whereas saturated lipids are usually very resistant to oxidative attack, fatty acids containing one or more double bonds (polyunsaturated fatty acids, PUFAs) readily participate in radical chain reactions. Lipid peroxidation reactions can be initiated by an attack of hydroxyl radicals on PUFAs (I):



Lipid radicals $R_1-HC^\bullet-R_2$ can then react with further lipid radicals and thus halt peroxidation reactions. However, lipid radicals can also react with molecular oxygen. Molecular oxygen O_2 is a hydrophobic molecule that concentrates into the interior of membranes. Reaction with lipid radicals leads to formation of lipid peroxy radicals (II):



Peroxy radicals are highly reactive and can absorb hydrogen atoms from other fatty acids resulting in propagation of lipid peroxidation reactions (III):



The lipid radical formed can then again react with O_2 to form another peroxy radical and thus the chain reaction of lipid peroxidation can continue. Lipid hydroperoxides can however be reduced by the enzyme PH-GPx (Imai et al., 2003), which is an important factor for the termination of lipid peroxidation chain reactions.

Lipid radicals can also be formed by reaction of lipids with perhydroxyl radicals (HO_2^\bullet), the protonated form of superoxide radicals (IV):



The superoxide radical anion by itself is insufficiently reactive and furthermore should not enter the hydrophobic membrane due to the negative charge. The pK_a of superoxide is approximately 4.8 (De Grey, 2002), therefore only a very small proportion of superoxide is protonated at physiological conditions. However the high reactivity as well as the ability to enter membranes make perhydroxyl radicals an important candidate for initiation of lipid peroxidation.

Perhydroxyl radicals can also stimulate peroxidation reactions by reaction with pre-formed lipid hydroperoxides (V):

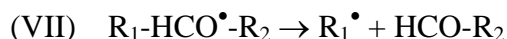


In vitro studies of lipid peroxidation suggest a pivotal role for transition metal ions in lipid peroxidation. Whether hydroxyl radicals generated by Fenton chemistry or rather some yet unidentified oxo-iron species are directly involved is still a matter of debate. Nevertheless, lipid peroxidation reactions can be greatly accelerated by Fenton systems containing e.g. copper or iron ions (Stohs and Bagchi, 1995).

Lipid peroxides can decompose to form various aldehydes and ketones which is

INTRODUCTION

accelerated by transition metal ions [(VI) and (VII)]:



The main products of lipid decomposition are malondialdehyde MDA and 4-hydroxynonenal HNE. These aldehydes both exhibit cytotoxic properties.

MDA can undergo toxic condensation reactions with amino residues in DNA bases and proteins. Mutagenic lesions from attack of MDA on guanine bases have been described (Benamira et al., 1995). Furthermore, MDA displays neurotoxic properties (Keller and Mattson, 1998).

Toxicity of HNE seems to be even higher than that of MDA, although its levels are smaller than those of MDA due to more structural requirements of PUFAs for HNE formation. HNE is formed during peroxidation of n-6 PUFAs such as linoleic and arachidonic acid (Pryor and Porter, 1990; Esterbauer et al., 1991). HNE can undergo several reactions that are supposedly cytotoxic. As is the case with MDA, HNE can react with guanine bases in nucleic acids and exhibits genotoxic properties (Eckl et al., 1993). Moreover, HNE can react with membrane proteins like Na⁺/K⁺-ATPases and Ca²⁺ channels (Siems et al., 1996; Lu et al., 2002) leading to disturbance of cell membrane potential and ion homeostasis, it can impair metabolic enzymes like pyruvate dehydrogenase and α -ketoglutarate dehydrogenase (an enzyme of the Krebs cycle) (Humphries and Szweda, 1998), damage mitochondria, inactivate cytoprotective NF κ B signaling (Camandola et al., 2000b) and increase AP-1 DNA binding (Parola et al., 1998; Camandola et al., 2000a). In addition, HNE has been reported to mediate oxidative stress-induced apoptosis in PC12 cells (Kruman et al., 1997).

Like MDA, HNE can be transformed by aldehyde dehydrogenases to the carboxylic acid or by aldose reductase or alcohol dehydrogenase to the alcohol (Chen and Yu, 1996). Furthermore, glutathione seems to play an important role in HNE detoxification by forming conjugates either nonenzymatically or catalysed by glutathione S-transferases (Goon et al., 1993; Hartley et al., 1995) (see also 1.1.3.2.2, page 10).

Nucleic acid oxidation

Oxidative damage to DNA and RNA can either affect the bases or the sugar residues. Oxidative attack on bases can be mutagenic, whereas oxidation of the sugar leads to strand breaks. Desoxyribose is much more prone to oxidation compared to ribose, and

among the bases, guanine is most sensitive towards oxidation due to its electron-rich amino moieties. The most abundant product of DNA base oxidation is 8-hydroxyguanine, which is often used as a marker for oxidative stress. Apart from direct attack of ROS on nucleic acids, bases can also be oxidized via lipid peroxidation products as described above.

Oxidatively modified DNA can be repaired either by base excision and resynthesis from the opposite strand or by recombination synthesis during replication. However, repair mechanisms fail to repair all defects so that oxidative DNA damage usually accumulates with age (Piperakis et al., 1998; Mendoza-Nunez et al., 2001). As a consequence, accumulation of DNA defects leads to expression of dysfunctional proteins that finally may result in cell death. Cell death can also result from activation of p53 by DNA damage followed by apoptotic signaling (see 1.1.3.5, page 15).

Protein oxidation

Oxidative protein modifications include a wide variety of reactions depending on the type of amino acids that are affected: sulfur-containing moieties in cysteine and methionine are easily oxidized to disulfides or sulfoxides respectively, basic amino acids arginine and lysine can be oxidized to aldehydes, aromatic rings in amino acids can be oxidized or nitrated and aliphatic carbon atoms can be oxidized to alcohols. Apart from direct attack of ROS, proteins can also be oxidatively modified by lipid peroxidation products or by sugars and aldehydes like glyoxal and methylglyoxal that lead to formation of advanced glycation end products AGEs (Münch et al., 1997).

The only repair mechanisms for proteins identified so far are restoration of cysteine thiol groups from disulfides, e.g. by thioredoxin, and restoration of methionine from sulfoxides by methionine sulfoxide reductase (Stadtman, 2004). Whether further mechanisms of protein oxidation repair exist is a matter of further research. However, oxidized proteins can be removed by proteolysis via the proteasome complex.

1.1.3.4 Intracellular signaling and transcription factors activated by ROS

Increased levels of ROS can either induce adaptive responses or elicit cell death. Often, mild oxidative stress leads to activation of antioxidant responses and resistance to higher ROS levels. By which mechanisms this adaption takes place is still under investigation, but there is growing evidence that ROS can activate discrete signaling pathways and gene transcription patterns.

INTRODUCTION

Intracellular signaling triggered by ROS is very complex [reviewed in (Finkel, 1998; Dalton et al., 1999; Allen and Tresini, 2000)]. One of the most extensively studied intracellular signaling cascades activated by ROS is the MAP-kinase pathway. Four MAP kinase subfamilies have been identified to date: ERK, JNK/SAP kinase, p38 kinase and big MAP kinase pathways. All these pathways contain redox-sensitive sites (Lo et al., 1996). Although exceptions are known, antioxidant compounds inducing ERK isoforms often elicit cytoprotective signaling, whereas oxidative stress activates JNK, p38 and big MAP kinase pathways that mostly result in cell death signaling. MAP kinases lead to phosphorylation of transcription factors c-jun and c-fos thereby affecting gene transcription, e.g. by the transcription factor AP-1 (see below).

Several transcription factors can be affected by ROS [reviewed in (Sun and Oberley, 1996; Sen and Packer, 1996; Dalton et al., 1999)], of which the most extensively studied are NF- κ B and AP-1 (Meyer et al., 1994; Muller et al., 1997). Upon activation, NF- κ B enters the nucleus, binds to DNA and can activate the transcription of several target genes like cytokines, growth factors and iNOS but also antioxidant genes like Mn-SOD and γ -glutamylcysteine synthetase, an enzyme involved in glutathione synthesis (White et al., 2000; Yang et al., 2001; Lee et al., 2003a).

AP-1 is a transcription factor that can be activated by prooxidant hydrogen peroxide but also by antioxidants. AP-1 is a dimer of two proteins, c-fos and c-jun, existing either as a inactive homodimer of phosphorylated c-jun or an active heterodimer of c-jun and c-fos. Therefore, activation of AP-1 can be due to either increased synthesis of AP-1 components or changes in phosphorylation state. Furthermore, binding of AP-1 to DNA is redox-sensitive via cysteine residues in the DNA-binding domains of c-jun and c-fos: thioredoxin increases DNA binding whereas oxidation decreases DNA binding (Abate et al., 1990). Thus, AP-1 corresponds not only to oxidative stress but also to certain antioxidants. A consensus sequence for AP-1 binding has been found in the antioxidant response element (ARE). Among the genes containing ARE sequences are glutathione-S-transferase and the amyloid precursor protein APP (La Fauci et al., 1989; Trejo et al., 1994), suggesting that APP expression can be modulated by ROS, which may be important in the pathogenesis of Alzheimer's disease.

Despite the above considerations, it has to be noted that the induction of NF- κ B or AP-1 transcriptional activity by ROS is cell-type dependent, furthermore DNA binding is negatively affected by oxidation. In addition, various intracellular factors as well as

DNA binding motifs contain thiol groups and are thus redox-sensitive (Hayashi et al., 1993). Hence the overall effects of ROS on gene transcription can be very variable. Moreover, whether NF- κ B or AP-1 activation exert an overall cytoprotective or cytotoxic effect is difficult to elucidate (Kamata and Hirata, 1999; Leong and Karsan, 2000; Shaulian and Karin, 2001; Das and Maulik, 2003; Ameyar et al., 2003; Gaur and Aggarwal, 2003).

1.1.3.5 Role of ROS in cell death

Damage of cellular components by ROS and insufficient repair mechanisms usually lead to a cell death response. Whether cells die by apoptosis or necrosis is largely dependent on the severity of insult: the apoptotic process, which is an energy-consuming process, requires some residual functionality of cellular proteins and ATP (Richter et al., 1996), whereas severe oxidative damage with destruction of cellular integrity and dissipation of ATP levels usually elicits necrotic cell death (Leist et al., 1997; Eguchi et al., 1997).

Apoptosis

Apoptotic cell death (Kerr et al., 1972) is different from necrotic cell death (see below) in that cells undergo an active suicidal program which leads to degradation of cellular components and release of apoptotic bodies without excretion of intracellular components. Apoptotic bodies are finally taken up by macrophages. Surrounding cells are usually not much affected by this process. Apoptosis is an essential part of normal development, homeostasis and elimination of damaged cells, but it can be disturbed under pathological conditions, e.g. in Alzheimer's disease, where neurons increasingly die by apoptosis (Su et al., 1994), or in neoplastic diseases, where apoptosis is impaired leading to abnormal growth of cells.

In recent years, mitochondria have gained the center of attention in apoptosis research [reviewed in (Susin et al., 1998; Tatton and Olanow, 1999; Eckert et al., 2003b)]. Figure 1.2 illustrates major apoptotic signaling pathways triggered by or associated with increased ROS levels.

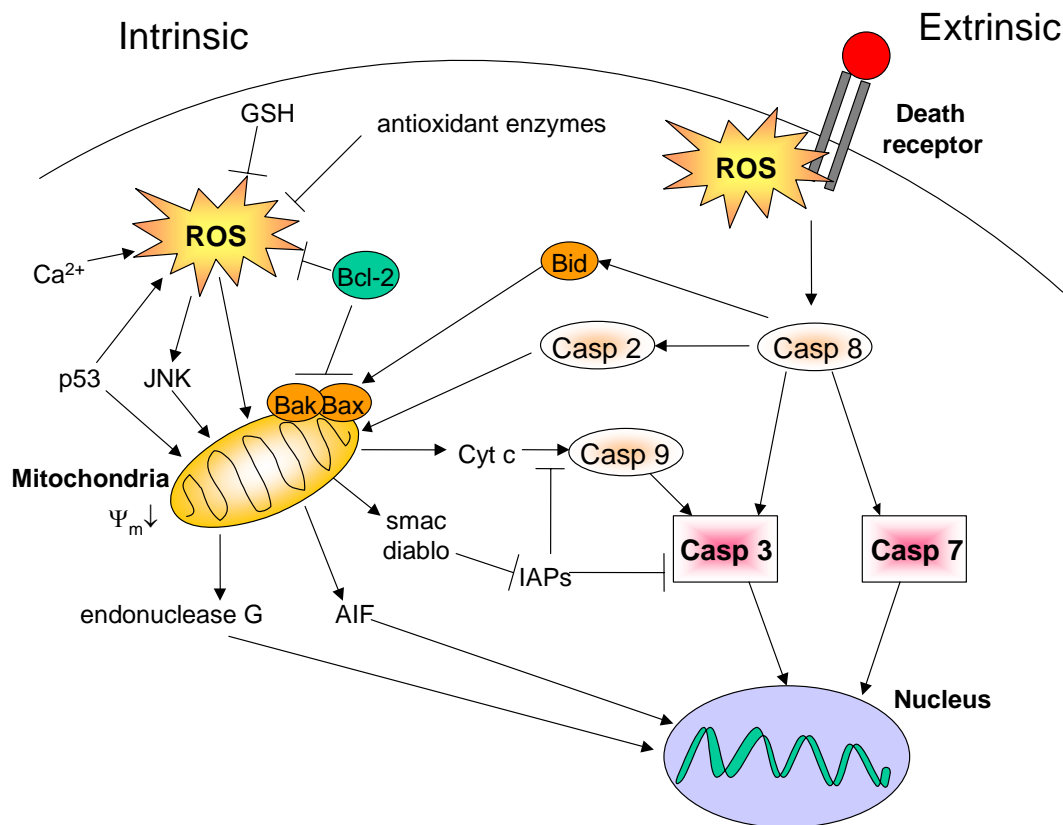


Figure 1.2: Major pathways in ROS-induced apoptotic signaling.

Abbreviations: AIF = apoptosis inducing factor, Casp = caspase, Cyt c = cytochrome c, GSH = reduced glutathione, IAPs = inhibitor of apoptosis proteins, JNK = c-jun amino terminal kinase pathway.

Activation of caspases – cysteine-dependent aspartyl proteases that lead to degradation of cellular components as characteristic feature of apoptosis – is in most cases at the center of the apoptotic process. Apoptotic signals can be derived from extracellular (“extrinsic pathway”) or intracellular sources (“intrinsic pathway”). Oxidative stress can cause apoptotic signaling (Buttke and Sandstrom, 1994), where several pathways can be activated: either direct oxidative toxicity to mitochondria can cause mitochondrial dysfunction associated with decreased mitochondrial membrane potential Ψ_m and release of mitochondrial factors initiating apoptotic signaling, or oxidative stress can activate or modulate death receptor signaling via Fas receptors (Bajt et al., 2002; Devadas et al., 2003). Several ROS can sensitize opening of the mitochondrial permeability transition pore, which is redox-regulated by mitochondrial glutathione levels [reviewed in (Zoratti and Szabo, 1995)]. Also, apoptotic signaling via p53 leads to ROS formation (Polyak et al., 1997). Consequently, overexpression of anti-oxidant enzymes like Cu/Zn-superoxide dismutase was able to prevent apoptosis (Greenlund et al., 1995; Przedborski et al., 1996), whereas underexpression pro-voked apoptosis (Troy

and Shelanski, 1994). The antiapoptotic effect of Bcl-2 has also been attributed to antioxidant properties (Garcia et al., 1992; Hockenbery et al., 1993; Kane et al., 1993; Steinman, 1995).

But ROS are not only initiators but also features of apoptosis: mitochondrial dysfunction can lead to increased leakage of electrons from the respiratory chain resulting in increased ROS formation (Zamzami et al., 1995).

ROS also seem to be involved in excitotoxicity caused by glutamate stimulation in neurons. Extracellular glutamate leads to activation of NMDA and/or AMPA/kainate receptors, rise in intracellular Na^+ and Ca^{2+} levels and several metabolic consequences. As stated in 1.1.3.1 (page 5 et seqq.), elevated Ca^{2+} levels result in increased production of ROS via nitric oxide synthases, phospholipase A activation and mitochondrial calcium overload. All these mechanisms can induce ROS accumulation and elicit cell death signaling.

Necrosis

In contrast to apoptosis, necrosis occurs under severe tissue damage that leads to disruption of cell membrane integrity, swelling of cells and organelles, rupture of the cell membrane and release of intracellular factors. Neighboring cells are affected and a local inflammatory reaction is initiated. Necrosis has been shown to occur under ischemic conditions or mechanic trauma. Also, severe oxidative stress causes necrosis (Lemasters et al., 1998). And vice versa, necrosis can be accompanied by increased ROS formation due to the release of transition metal ions from proteins or inflammatory reactions.

In summary, ROS are major activators but also features of different types of cell death. However, it is often difficult to elucidate the role of ROS *in vivo*, i.e. whether ROS are causal agents or only bystanders of cell death.

1.2 Oxidative stress in brain aging

1.2.1 Aging theories

Aging seems to be a very complex, multicausal phenomenon, which can not be fully explained by any single theory. Aging can be regarded as a time-dependent irreversible accumulation of cell damage or dysfunction that leads to morbidity and mortality. Inherent properties of cells in multicellular organisms encoded by the genome as well as epigenetic and environmental factors seemingly contribute to the aging phenomenon.

On a cellular basis, replicative senescence, i.e. the inability of cells to undergo further mitotic divisions, has been attributed to telomere shortening. Telomeres are DNA base sequence repeats at the ends of chromosomes which are shortened during each mitotic cell division. Shortened or disrupted telomeres mimic DNA damage and result in p53 activation leading to cell-cycle arrest (Blackburn, 2000). Telomere shortening can be counteracted by expression of telomerase, an enzyme that restores telomeres. Telomerase expression has been shown to confer immortality to cells that would otherwise undergo replicative senescence (Bodnar et al., 1998). However, telomerase-independent pathways of initiating cellular senescence must exist, since rodent cells in culture possess exceptionally long telomeres, express telomerase and keep telomere length but nevertheless have limited proliferative capacity *in vitro* (Blasco et al., 1997).

The somatic mutation theory of aging tries to explain the aging phenomenon by accumulation of spontaneous DNA mutations during life. As a result, protein expression or function becomes impaired which leads to age-associated defects and finally death (Corral-Debrinski et al., 1992). The mitochondrial genome seems to be especially vulnerable to DNA mutations since mitochondrial DNA, unlike nuclear DNA, is not protected by DNA-associated proteins like histones and is furthermore localized within the mitochondrial matrix, where ROS are continuously produced by electron leakage from the respiratory chain. Oxidative damage to mitochondrial DNA and impaired production of ATP have been suggested to play a role in age-associated degeneration (Miquel et al., 1980; Wallace et al., 1992).

Deficits caused by aging have also been tried to be explained by an attack of the immune system on body cells (Smith and Walford, 1977). Increased levels of autoantibodies have been detected in aged humans (Hijmans et al., 1984). It is therefore conceivable that elimination of cells by an overactive immune system leads to

insufficient restorage of cells and age-related deficits.

Evidence that genetic factors are involved in the aging phenomenon stems from the identification of genes or gene mutations that influence life span. Several factors have been identified in *C. elegans* and *Drosophila* [reviewed in (Finkel and Holbrook, 2000)]. In mammals, mice with a knockout of the *klotho* gene – coding for a protein with unidentified function – and p53 mutant mice display a shortened life span (Tyner et al., 2002). Conversely, a mutation in the *shc* gene encoding an intracellular adaptor protein that controls oxidative stress response increases the life span of mice (Migliaccio et al., 1999). Unknown genetic factors accelerate aging in the senescence-accelerated mouse model (Troen, 2003). Overexpression of growth hormone is associated with decreased life span (Bartke et al., 1994), whereas growth hormone deficiency in dwarf mice prolongs life span (Brown-Borg et al., 1996; Bartke et al., 1998). In addition, inactivation of the receptor for insulin-like growth factor IGF-1 slowed growth, but increased resistance to oxidative stress and prolonged life span (Bartke et al., 2003). These observations point to a role for growth factor signaling and metabolic activity in aging.

Candidate genes that determine life span in humans include the major histocompatibility complex (Caruso et al., 2001), the apolipoprotein E (Smith, 2000) and the angiotensin-converting-enzyme (Schachter et al., 1994). It is noteworthy that a pronounced difference in life expectancy between males and females exists in humans, which is also found in some other mammalian species (Smith and Warner, 1989). Which factors determine these gender differences have yet to be explored.

1.2.2 The free radical theory of aging

The free radical theory of aging, as initially proposed by Denham Harman in the 1950s (Harman, 1956), is compelling in that it can unite several aspects of aging. In its original version, it assumes that free radical reactions, modified by genes and environment, are the single basic cause of aging. These free radical reactions can arise from ionising radiation, non-enzymatic reactions and from enzymatic reactions, particularly those employed in the reduction of molecular oxygen to water by the respiratory chain in mitochondria.

There is a vast body of evidence arguing in favour of this hypothesis, which is often thought of synonymously with the rate-of-living hypothesis that was already proposed in the 1920s (McCoy et al., 1935). Life span seems to be correlated with metabolic rate

or even better with mitochondrial radical formation: a high metabolic rate, consistent with increased mitochondrial respiration and increased radical formation, was associated with a low average life span (Ku et al., 1993; Sohal et al., 1995). Consequently, caloric restriction can efficiently increase life span of rodents (Sohal and Weindruch, 1996) and possibly also of primates (Lane et al., 2001), and is associated with reduced ROS formation (Tian et al., 1995; Gabbita et al., 1997; Lopez-Torres et al., 2002). Antioxidants, antioxidant enzyme mimetics and similarly overexpression of antioxidant enzymes have been shown to increase the life span of several different species (Harman, 1981; Orr and Sohal, 1994; Stoll et al., 1997; Melov et al., 2000), whereas mutation of antioxidant genes reduced life span (Taub et al., 1999). Furthermore, reduced levels of antioxidants as well increased markers of oxidative stress were found in aged animals (Chen et al., 1989; Ravindranath et al., 1989; Carney et al., 1991) and humans (Ando et al., 1995; Yang et al., 1995; Shemyakov and Mikhailova, 2003).

It must however be noted that species differences (Igarashi and Satoh, 1989; Sohal et al., 1990) as well as a different vulnerability of various organs exist (Carrillo et al., 1992). Therefore, findings from aging studies in insects or even rodents should not be uncritically transferred to the situation in humans. Similarly, conclusions from studies on e.g. changes in oxidative parameters in blood as to the actual situation in a specific organ should be drawn carefully.

1.2.3 A role for oxidative stress in brain aging?

The brain is considered to be especially vulnerable towards oxidative stress due to several reasons (Halliwell, 1992):

(i) The brain operates at a high metabolic rate. It accounts for less than 2 % of the body weight, but consumes 20 % of the basal oxygen uptake. High oxygen consumption via mitochondria is linked to leakage of electrons along the respiratory chain with subsequent radical formation.

(ii) There is a high content of polyunsaturated fatty acids PUFAs in brain cell membranes. PUFAs are especially prone to undergo lipid peroxidation reactions (see 1.1.3.3, page 10 et seqq.) resulting in formation of cytotoxic aldehydes MDA and HNE. Furthermore, some brain regions contain high levels of iron, which is for example contained in neurotransmitter-synthesizing enzymes tyrosine and tryptophane hydroxylase. Upon tissue injury, free iron ions can be released and give rise to radical

formation derived from Fenton chemistry (see 1.1.1, equation (I), page 4).

(iii) Neuronal activity is linked to signaling and metabolic pathways that favour formation of ROS. The consequences of increased intracellular calcium levels during neuronal depolarization have been described in 1.1.3.1. (page 5). Furthermore, the brain contains relatively high levels of nitric oxide that can give rise to formation of highly reactive peroxynitrite. Also, catecholamine metabolism involves increased ROS formation: superoxide can be generated from semiquinone formation, and hydrogen peroxide is released as by-product of catecholamine degradation by monoaminoxidase (Cadenas and Davies, 2000).

(iv) The brain possesses only relatively low levels of antioxidant defenses. Catalase activity is extremely low in brain tissue, and glutathione peroxidase and superoxide dismutase show low activity compared with other organs like liver, heart and kidney (Marklund et al., 1982).

(v) Neurons as postmitotic cells can not divide to substitute for dying cells. Therefore, it was long thought that tissue damage can not be reconstituted by neuronal cell growth. Although stem cells, which have the potential to differentiate into neurons and glial cells, have been identified in certain rodent brain areas [(Altman, 1962), recently reviewed in (Scheffler et al., 1999; Cameron and McKay, 1999)], it is questionable whether this actually occurs to a relevant extent in vulnerable brain regions *in vivo*.

While (i) to (iii) contribute to increased ROS formation, (iv) and (v) limit regeneration of brain cells. As a consequence, increased levels of oxidative stress during aging can be especially detrimental to brain tissue.

Experimental evidence that oxidative stress is involved in brain aging comes from studies that markers of oxidative damage are increased in aged animals. Mostly rodents have been studied, however results are controversial (Ceballos-Picot et al., 1992; Bonnes-Taourel et al., 1993; Mo et al., 1995; Leutner et al., 2001; Hamilton et al., 2001).

Since increased oxidative damage can be a consequence of ROS accumulation due to impaired detoxification, antioxidant factors have also been studied, again, with conflicting results. Reduced glutathione has mainly been reported to be reduced in aged mouse brains (Chen et al., 1989; Sasaki et al., 2001), but increased glutathione levels have also been found (Hussain et al., 1995). Similarly conflicting results have been obtained for activities of antioxidant enzymes, which were reported to be either

INTRODUCTION

increased (Ceballos-Picot et al., 1992; Leutner et al., 2001), decreased (Mo et al., 1995) or unchanged (Cardozo-Pelaez et al., 1999), and moreover results were not uniform for different antioxidant enzymes (Ceballos-Picot et al., 1992; Leutner et al., 2001).

Obviously, brain aging is accompanied by various adaptive changes in oxidative stress parameters which are however not uniform under different conditions. The conflicting results may be due to differences in methodology, differences between brain regions (Benzi et al., 1988; Carrillo et al., 1992), animal strains or animal maintenance. This necessitates a careful analysis of changes in oxidative stress parameters in each individual animal strain under the respective housing conditions when this animal strain is the genetic background for studies of further age-related degenerative processes in transgenic mice. Furthermore, the relevance of studies on rodent brains with respect to degenerative processes in the aging human brain remains to be elucidated.

1.3 Oxidative stress and Alzheimer's disease

1.3.1 Alzheimer's disease – clinical symptoms, pathology and risk factors

1.3.1.1 Clinical picture of Alzheimer's disease

When Alois Alzheimer described the symptoms and pathology of a quickly progredient dementia in a female patient in 1907 (Alzheimer, 1907), he probably couldn't have imagined the impact that the disease named after him would receive at the end of the century. Alzheimer's dementia (AD) is a neurodegenerative brain disease and the most common form of dementia among the elderly. From epidemiological studies it can be estimated that about 60 to 70 % of the dementia cases can be attributed to AD (Ritchie and Lovestone, 2002). In Western countries, AD represents one of the most frequent causes of death among the elderly – ranking 7th after cardiovascular diseases, cancer, stroke, chronic diseases of the lower respiratory tract, pneumonia and diabetes (Anderson and Smith, 2003). Since AD is an age-related disease, during Alzheimer's lifetime only few persons were able to live to an age where the risk to develop the disease is greatly increased. But as life expectancy in industrialized countries has been ever increasing, AD poses and will pose an ever heavier burden on societies.

Alzheimer's dementia is clinically characterized by progressive loss of intellectual function, memory, language skills as well as spatial and temporal orientation. This is accompanied by loss of activities of daily living, mood and personality changes, depression, agitation, anxiety or hallucinations and problems recognizing friends and family members. From the time of diagnosis, the average disease duration is approximately 5 to 8 years (Molsa et al., 1986; McDonnell et al., 2001; Wolfson et al., 2001). In the late stages of the disease, patients are completely disorientated, confined to bed and malnourished due to difficulty with swallowing. Most patients die from pneumonia (Wada et al., 2001).

Diagnosis of AD is complex and based on an exclusion of other causes of cognitive symptoms – e.g. vitamin B12 deficiency, depression, infectious CNS diseases, multiple sclerosis or brain tumors. Furthermore, other types of dementia like vascular dementia must be excluded. Several cognitive tests can be used to quantify the extent of cognitive impairment (McDougall, 1990; Storey et al., 2002). The most widely used are the Mini Mental Status Examination MMSE – a short test of cognitive abilities giving a score

from 30 (no cognitive impairment) to 0 (severe dementia) – , the global deterioration scale (GDS) – where a range of symptoms is judged by the physician – or the Alzheimer’s Disease Assessment Scale (ADAS) – a comprehensive analysis of cognitive and non-cognitive symptoms. Furthermore, morphological and functional imaging of AD brains can be used for diagnosis and monitoring the progress of AD. In AD patients, brain atrophy – especially in the medial temporal lobe – due to neurodegeneration can be detected by imaging methods (Scheltens and Korf, 2000), and glucose utilization in the temporal lobe – as measured by 18-F-desoxy-glucose positron emission tomography (PET) – is decreased (Herholz, 2003).

1.3.1.2 AD neuropathology

However, the definite confirmation of the diagnosis of AD can only be derived from pathological brain analysis after the death of the patient. Macroscopically, AD brains are characterized by reduced brain weight, atrophy of several brain regions, enlarged sulci and liquor ventricles. Degeneration affects several brain regions to a different extent, of which hippocampal structures and – at later stages – neocortical areas are severely degenerated. The neuropathological hallmarks of Alzheimer’s disease are increased numbers of senile plaques – extracellular 5-200 μm aggregates that are composed of amyloid beta $\text{A}\beta$ peptides (Glenner and Wong, 1984) and surrounded by dystrophic neurites – and intracellular neurofibrillary tangles that are composed of hyperphosphorylated tau protein (Grundke-Iqbal et al., 1986; Goedert et al., 1992) (see Figure 1.3). 80 % of AD cases also have vascular deposition of $\text{A}\beta$ in cerebral blood vessels (Masters et al., 1985a). Furthermore, plaque-only AD without the presence of neurofibrillary tangles – but with hyperphosphorylated tau – has also been described (Terry et al., 1987; Tiraboschi et al., 2004c).

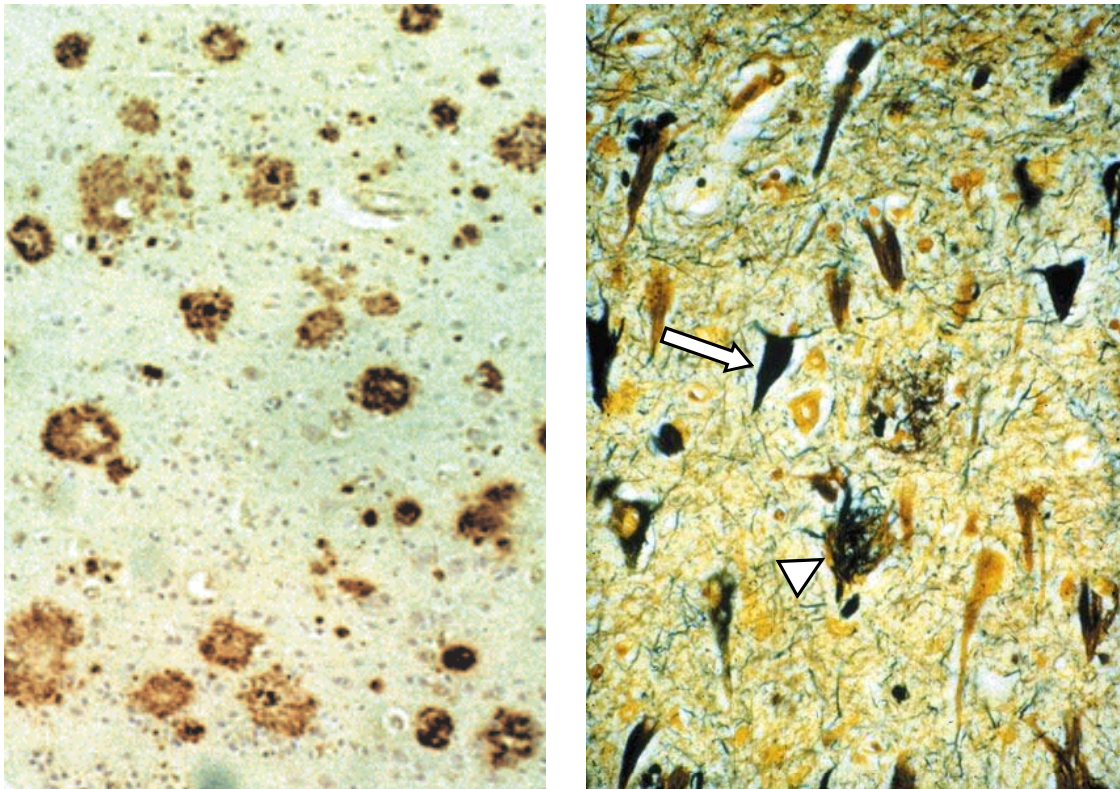


Figure 1.3: Neuropathological hallmarks of Alzheimer's disease: senile plaques (left panel) and neurofibrillary tangles (right panel).

Left panel: Staining of senile plaques with anti-A β antibody [taken from (Steiner and Haass, 2000)].

Right panel: Silver staining of intracellular neurofibrillary tangles. Hyperphosphorylated and abnormally aggregated tau protein is found in pyramidal neurons (arrow) and in neurites surrounding amyloid plaques (arrowhead) [taken from (Mattson, 2000)].

The presence of plaques and tangles is, however, not specific for AD. Even the brains of healthy subjects without clinical symptoms of dementia before death can contain several plaques, which are however largely of the diffuse type in contrast to the predominantly senile type of plaques in AD brains. Numbers and density of plaques and tangles are higher in AD brains and show a typical distribution in certain brain regions. The number of plaques does not correlate with clinical severity of AD (Braak and Braak, 1991; Nagy et al., 1995; Gomez-Isla et al., 1997; Giannakopoulos et al., 2003; Guillozet et al., 2003), but the distribution and quantity of neurofibrillary tangles gives a good correlate with the clinical cognitive state, and it moreover correlates with measures of neurodegeneration. Braak and Braak have staged the neurodegenerative progress in AD according to the distribution of neurofibrillary degeneration (Braak and Braak, 1991). In stages I and II, only entorhinal structures are affected, without cognitive impairment of the patient. In stages III and IV, hippocampal structures are also affected and mild cognitive impairment is present. In stages V and VI, neurodegeneration proceeds to

cortical areas which represents the full clinical picture of AD.

Despite extensive research in the field, no causal therapies exist until today – almost a hundred years since Alzheimer’s case report. The only drugs approved so far are inhibitors of acetyl choline esterase and Memantine, a NMDA-receptor antagonist. The clinical efficacy of these substances is limited, as none of these drugs is able to halt the progression of AD, only a reduction in the rate of progression is achieved, which retards progression for about one year. It is therefore essentially necessary to understand more about the pathology leading to the development of AD for generation of new drug targets that will finally enable a causal therapy of AD.

1.3.1.3 Genetic and non-genetic risk factors for AD

Identification of factors that contribute to the pathology of AD comes from epidemiological as well as genetic studies. Alzheimer’s disease can be classified into two different forms, rare early-onset forms where the disease onset is at an age younger than 60 years, and the vast majority of cases where onset occurs at an age over 60. Both forms of AD show the same clinical symptoms and neuropathology. In the sporadic form of the disease, several factors have been found that increase the risk to develop the disease, but – unlike familial AD (FAD) mutations – do not necessarily lead to development of AD. Aging is by far the most important risk factor for AD, but also the apolipoprotein E4 allele and female gender predispose to the development of AD (Figure 1.4). Genetic studies in FAD patients have identified mutations in the genes encoding for the amyloid precursor protein APP on chromosome 21 and for the presenilins PS1 and PS2 on chromosomes 14 and 1 that cause an autosomal inherited form of AD with 100 % penetrance. Onset of clinical symptoms in FAD patients is often much earlier in life, especially for carriers of PS1 mutations¹ (see Figure 1.4).

¹ The progressive loss of cognitive abilities of a patient suffering from early-onset FAD has been very authentically portrayed in the movie “Claire – Se souvenir des belles choses” by Zabou Breitman (www.sesouvenirdesbelleschoses.com).

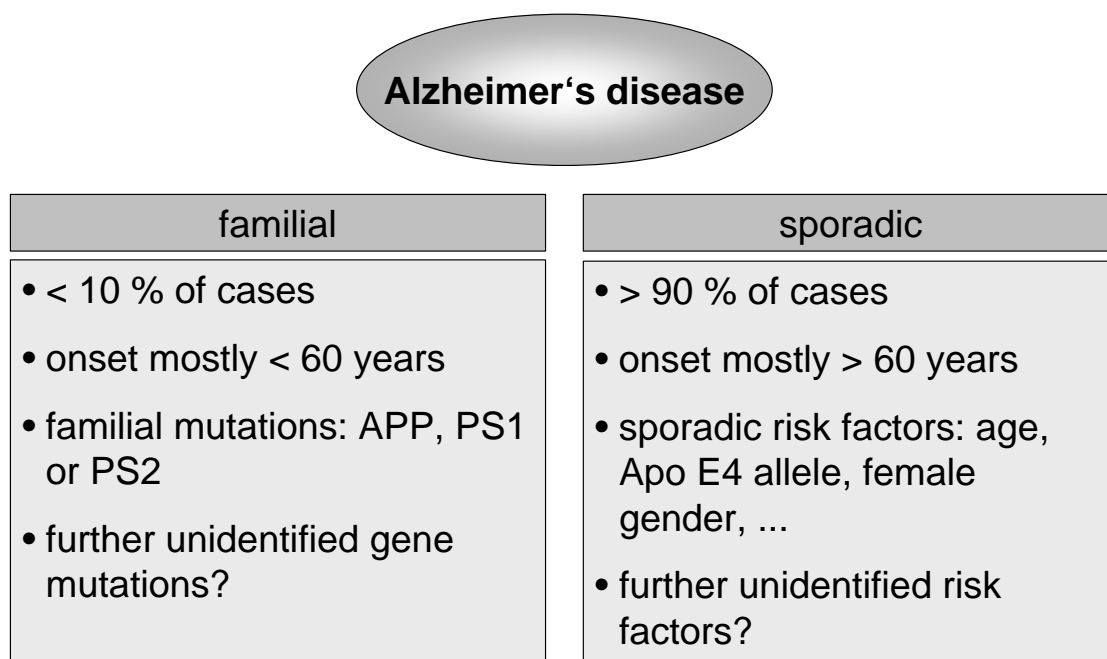


Figure 1.4: Classification of Alzheimer's disease.

Familial AD cases represent the minority of cases, which usually show an early onset at an age younger than 60 years. Mutations in the amyloid precursor protein APP or the presenilins PS1 and PS2 have been identified as causal factors. Sporadic AD cases constitute the majority of cases, where age of onset is usually above 60 years. Aging is the most important risk factor for sporadic AD, but also the apolipoprotein E4 allele and female gender increase the risk to develop AD. However, yet unknown genetic mutations and risk factors probably exist in both forms of AD.

1.3.2 Risk factors for sporadic AD

The most important risk factor for sporadic AD is aging. Several epidemiological studies have evidenced that the prevalence of AD is greatly increased among aged people, and prospective studies have confirmed that the incidence of AD is higher in aged subjects [reviewed in (Jorm et al., 1987; Gao et al., 1998; Fratiglioni et al., 1999)]. For example, prevalence of AD is 20 % in people aged 80 to 89 years and 40 % in subjects aged more than 90 years (Katzman, 1976). The incidence rates for AD increase from 0.07 % in the age group of 65 to 69 years to 6.6 % in persons aged 90 years or older (Letenneur et al., 1994), i.e. the risk for a person aged 90 years or older to develop AD within 1 year is 100 times higher than the risk of a person at the age of 65 to 69.

Genetic studies have furthermore elucidated a role for apolipoprotein E alleles in the etiology of AD. The gene for apolipoprotein E (Apo E) is encoded on chromosome 19, with three different allelic variations: Apo E 2, 3 or 4. Carriers of apolipoprotein E4 alleles have a higher risk to develop AD (Saunders et al., 1993; Poirier et al., 1993; Corder et al., 1993; Strittmatter et al., 1993; Dal Forno et al., 1996), which shows a gene

dosage effect: risk is highest in homozygous and lowest in non-Apo E4 carriers. In contrast to Apo E4, Apo E2 alleles seem to exert a protective effect (Corder et al., 1994; Talbot et al., 1994; Locke et al., 1995; Polvikoski et al., 1995; Hyman et al., 1996; Lippa et al., 1997). The higher risk for AD conferred by the Apo E4 allele is probably due to a lower age at onset of AD, but not to an overall increased lifetime susceptibility (Khachaturian et al., 2004).

Several studies have reported a higher prevalence and incidence of AD in women compared to age-matched men (Jorm et al., 1987; Zhang et al., 1990; Fratiglioni et al., 1997; Andersen et al., 1999; Copeland et al., 1999; Ruitenberg et al., 2001), although these findings are still controversial (Rocca et al., 1998; Hebert et al., 2001). Women have an approximately 1.5- to 3-fold increased risk to develop AD compared to men. This risk is even higher in very old women aged more than 90 years (Fratiglioni et al., 1997). Furthermore, polymorphisms in the estrogen receptor alpha gene (Brandi et al., 1999; Ji et al., 2000) and aromatase (Iivonen et al., 2004) were in some studies associated with an increased risk for AD.

In addition to the above-mentioned risk factors, further genetic and life-style factors have been elucidated that modulate the risk to develop AD, although some are still controversially discussed. For example, some studies have shown an inverse correlation between education and risk of dementia: low education increased the risk to suffer from dementia up to twofold (Zhang et al., 1990; Karp et al., 2004). Furthermore, several studies have repeatedly reported that a history of head injury increases the risk for AD by about 1.5-fold (Fleminger et al., 2003).

Apart from Apo E status and female gender, further genetic factors have been identified that could contribute to the risk for AD, although confirmation of most of these factors is needed in further studies. Candidates are several oxidative stress-related genes like methionine synthase (Beyer et al., 2003), glutathione-S-transferase (Li et al., 2004b), oxidized LDL-receptor 1 (Lambert et al., 2003), myeloperoxidase (Reynolds et al., 1999; Leininger-Muller et al., 2003) as well as alpha-2-macroglobulin (Liao et al., 1998; Chen et al., 2004) and other inflammatory genes (McGeer and McGeer, 2001). Recently, an interaction of alpha-2-macroglobulin and myeloperoxidase polymorphisms leading to an increased risk for AD has also been reported (Zappia et al., 2004).

1.3.3 Familial AD mutations and the amyloid beta hypothesis

1.3.3.1 The amyloid precursor protein APP – physiological processing and effect of APP mutations

Research on Alzheimer's disease was boosted at the end of the 1980s by the discoveries (i) that the 39 to 42 amino acid amyloid beta peptide A β is the primary component of the senile plaques present in Alzheimer's disease patients (Glenner and Wong, 1984; Masters et al., 1985b; Iwatsubo et al., 1994), (ii) that A β is derived from its precursor protein APP encoded on chromosome 21 (Kang et al., 1987; Tanzi et al., 1987) and (iii) that mutations in the APP gene lead to the development of early-onset forms of AD in very few families worldwide (Levy et al., 1990; Chartier-Harlin et al., 1991; Goate et al., 1991; Van Broeckhoven et al., 1992; Mullan et al., 1992; Hendriks et al., 1992). Several other mutations in the APP gene have been discovered so far, all of which are within or proximate to the A β sequence and for most of which an increased processing of APP towards higher formation of A β or especially the 1-42 amino acid form of A β has been shown.

APP is a type I integral membrane protein with glycosylation sites on the extracellular C-terminus. Subcellular localization of APP is in the ER, trans-golgi network and the cell membrane, but it has recently also been identified in mitochondria in APP transgenic mice (Selkoe et al., 1996; Hartmann et al., 1997; Anandatheerthavarada et al., 2003). In general, APP can be processed by two distinctive pathways: either by α -secretase cleavage (Sisodia et al., 1990) yielding sAPP α and C83, which is cut intramembraneously by γ -secretase to release p3 and AICD (APP intracellular domain), or by β -secretase resulting in formation of sAPP β and C99, followed by γ -secretase cleavage with production of A β and AICD (Haass et al., 1993) (see Figure 1.5). The γ -secretase cut can take place at different amino acids in the A β sequence, which leads to formation of A β ₁₋₄₀ or A β ₁₋₄₂. The main species generated is A β ₁₋₄₀ with approximately 90%, while only 10% A β ₁₋₄₂ are formed (Seubert et al., 1992; Haass et al., 1992a).

INTRODUCTION

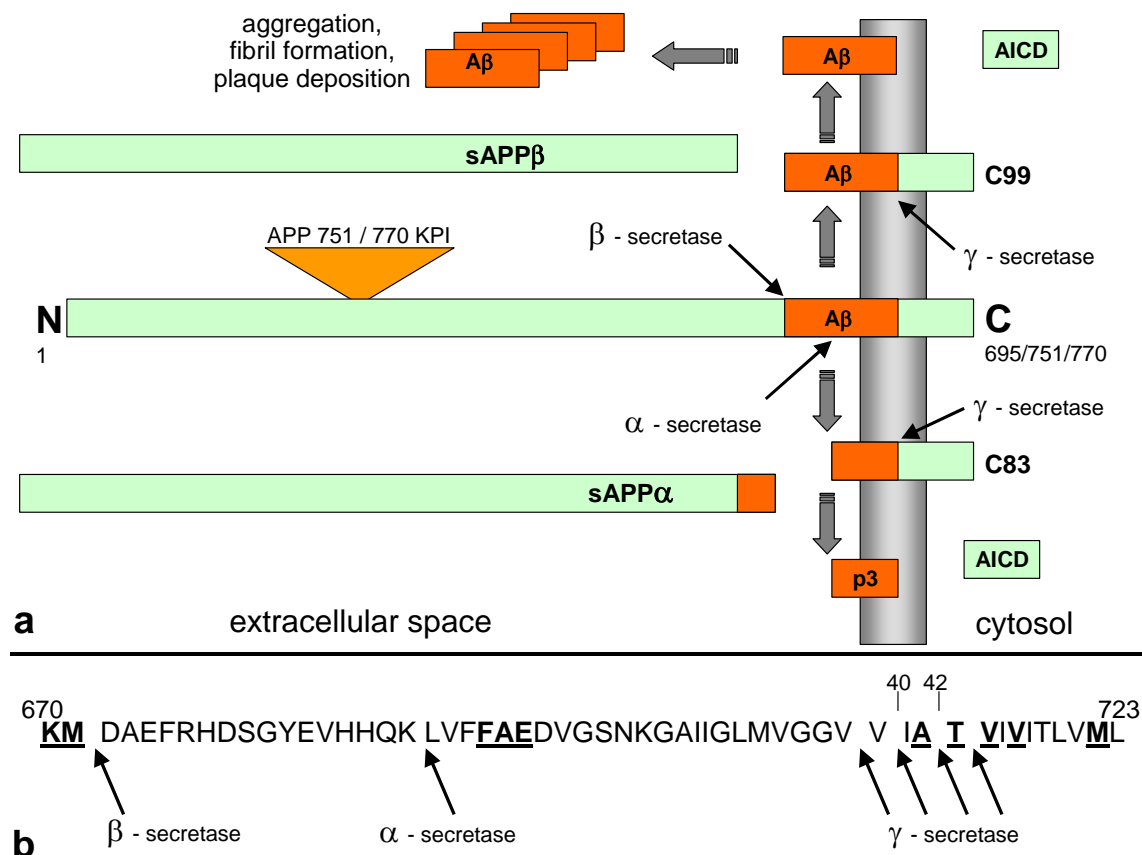


Figure 1.5: Formation of A β peptides from cleavage of APP

a. Structure of APP, cell membrane topology and cleavage by secretases. Cleavage of APP by α -secretase results in formation of N-terminal sAPP α and the 83 amino acid C-terminal peptide C83, which is subsequently cut by γ -secretase to release the p3 peptide and the APP intracellular domain AICD. β -amyloidogenic cleavage of APP is effected by β -secretase cleavage yielding sAPP β and the C99 peptide followed by γ -secretase cleavage resulting in formation of A β and AICD. The longer 751 or 770 amino acid isoforms of APP contain the kunitz protease inhibitor insert KPI.

b. Amino acid sequence of APP in the A β region. Bold and underline letters represent amino acids where FAD mutations have been identified. Of note, FAD mutations lie close to or within secretase cleavage sites. Amino acid numbering refers to the 770 amino acid isoform of APP.

α -secretase has not been unambiguously identified, but the metalloproteases ADAM10 and ADAM17 (TACE) have been suggested (Buxbaum et al., 1998b; Lammich et al., 1999), and *in vivo* evidence has been provided that moderate overexpression of ADAM10 in APP transgenic mice can increase α -secretase cleavage of APP (Postina et al., 2004). β -secretase (BACE) has recently been identified (Vassar et al., 1999; Yan et al., 1999; Sinha et al., 1999). Two homologs exist, BACE, which was first identified and is mainly expressed in brain, and BACE 2, which is expressed predominantly in peripheral tissues and only at low levels in brain (Bennett et al., 2000). BACE knockout mice show no apparent deficits (Luo et al., 2001; Roberds et al., 2001), while BACE transgenic mice show increased formation of A β (Mohajeri et al., 2004). γ -secretase activity is effected by a multiprotein complex containing the presenilins (Ray et al.,

1999; Xia et al., 2000; Yu et al., 2000).

APP is widely expressed throughout the body, in the central nervous system as well as in peripheral cells. The APP gene contains 18 exons, and different splice variants lead to formation of multiple isoforms (Hattori et al., 1997). In brain tissue, the most abundant forms are the isoforms containing 695, 751 and 770 amino acids (Sola et al., 1993). The 695 isoform is mainly localized in neurons, whereas glial cell express mostly the 751 isoform (Forloni et al., 1992; Shioi et al., 1995). The 751 and 770 amino acid isoforms contain the Kunitz protease inhibitor KPI region (Tanzi et al., 1988). Although still controversial [reviewed in (Rockenstein et al., 1995)], APP isoforms with KPI regions were found to be increased in brains from AD patients compared to controls in more recent publications (Tanaka et al., 1992; Zhan et al., 1995; Moir et al., 1998; Preece et al., 2004). Furthermore, processing of the two isoforms is different in that the 751 isoform is cleaved to yield more A β than the 695 isoform (Ho et al., 1996), which suggests a pathological role for the 751 isoform in AD pathogenesis.

Among the mutations in APP associated with early-onset FAD are mutations in amino acid 717 of APP (“London” mutation), i.e. close to the γ -secretase cleavage site, that lead to increased production of the longer A β ₁₋₄₂ species (Suzuki et al., 1994; Scheuner et al., 1996; Eckman et al., 1997), a double mutation at the β -secretase cleavage site in position 670/671 identified in a Swedish family leading to 6- to 8-fold higher production of total A β species (Citron et al., 1992; Mullan et al., 1992) and the “Dutch” mutation E693Q in vicinity of the α -secretase cleavage site, which leads to reduced α - and conversely increased β -secretase cleavage of APP (Haass et al., 1994). Furthermore, this mutation lies within the A β sequence and causes a structural change of A β thereby accelerating fibril formation (Levy et al., 1990; Wisniewski et al., 1991; Clements et al., 1993).

A β peptides tend to aggregate, which is more pronounced for the longer A β ₁₋₄₂ compared to A β ₁₋₄₀ (Pike et al., 1991b; Harper et al., 1997). A β ₁₋₄₂ seems to be the initial component giving rise to formation of amyloid plaques detected in the brains of AD patients (Iwatsubo et al., 1994). These findings and the observation that Down’s syndrome patients with trisomy 21, the chromosome that contains the APP sequence, develop Alzheimer’s disease neuropathology led to the development of the amyloid cascade hypothesis (Hardy and Higgins, 1992). It states that the development of AD is due to abnormal accumulation of A β in the brains of AD patients causing

INTRODUCTION

neurodegeneration and finally the clinical symptoms of dementia (Figure 1.6). Support for this hypothesis not only stems from the identification of FAD mutations that increase production of A β , but also from association of sporadic AD risk factors with increased A β production, which was shown for aging (Fukumoto et al., 2003; Fukumoto et al., 2004) and the Apo E4 allele (Schmechel et al., 1993; Olichney et al., 1996; Berg et al., 1998; Johnson et al., 1998; Lue et al., 1999; Ghebremedhin et al., 2001; Tiraboschi et al., 2004a).

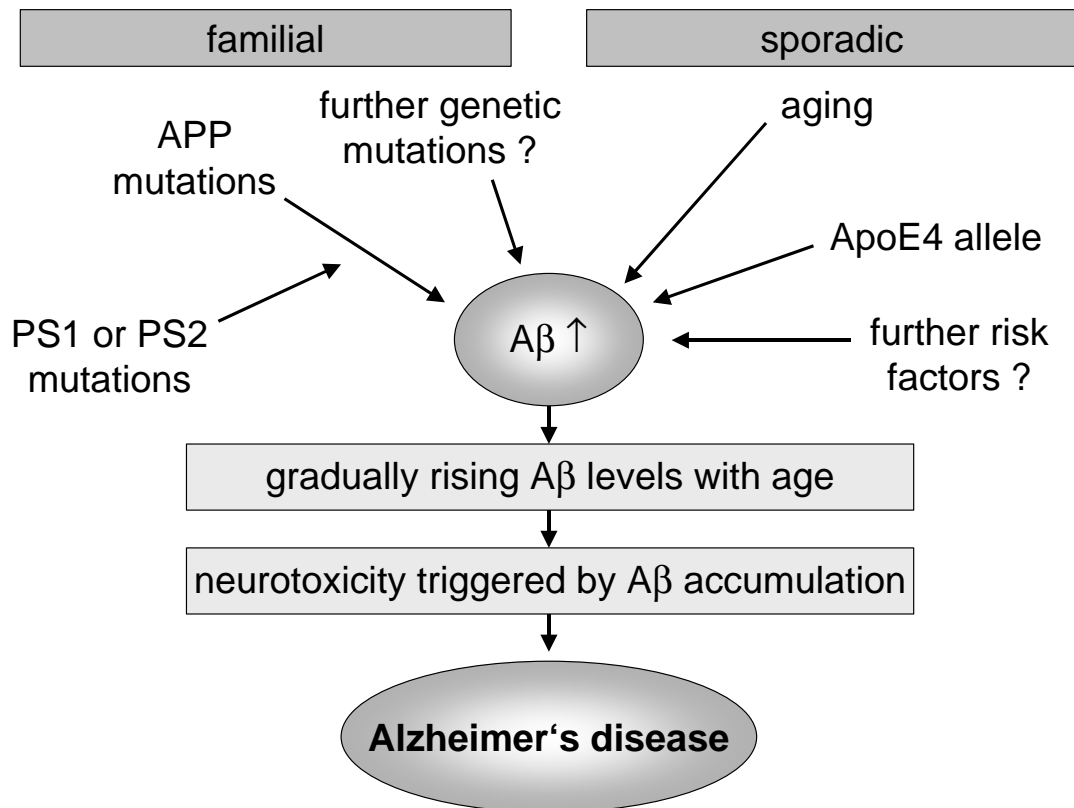


Figure 1.6: The amyloid cascade hypothesis of Alzheimer's disease.

As initially suggested by Hardy and Higgins (Hardy and Higgins, 1992), the accumulation of Amyloid beta peptide A β constitutes the central event in the pathogenesis of Alzheimer's disease. Either mutations in the APP gene or the presenilins PS1 or PS2 cause increased formation and aggregation of A β leading to accumulation of the peptide and neurotoxicity with the final clinical outcome of Alzheimer's disease. The hypothesis states that other genetic or sporadic risk factors for the disease similarly favour the accumulation of A β resulting in the same neurotoxic cascades as seen in the familial forms of the disease with the same clinical endpoint of Alzheimer's disease.

It must however be noted that criticism of the Amyloid cascade hypothesis has been put forward (Bishop and Robinson, 2002), which is mainly based on findings (i) that A β is produced in substantial amounts also under physiological conditions (Haass et al., 1992b), (ii) that neurotoxicity was not reproduced under all experimental conditions (Podlisny et al., 1992; Stein-Behrens et al., 1992) and (iii) A β plaques in AD brains do

not correlate with the severity of cognitive impairment (Braak and Braak, 1991; Nagy et al., 1995; Gomez-Isla et al., 1997; Giannakopoulos et al., 2003; Guillozet et al., 2003). Therefore, different toxicities of isoforms of A β (Zou et al., 2003), aggregation state (Pike et al., 1993) and subcellular localization might help to modify the amyloid beta hypothesis to better fit all experimental evidence (Hardy and Selkoe, 2002).

1.3.3.1.1 Putative physiological functions of APP

Physiological roles for APP and the derived species are not completely understood. Properties of APP that could play a role in cell signaling have been proposed, among these are a role of APP as a cell surface receptor (Kang et al., 1987), interaction of APP with G₀-proteins (Nishimoto et al., 1993) and a role of the APP intracellular domain (AICD) as a transcription factor regulating calcium homeostasis (Leissring et al., 2002). But also trophic functions especially of sAPP α (Mucke et al., 1994; Mucke et al., 1996; Bour et al., 2004) or a role for APP in cell-cell contacts are discussed (Ghiso et al., 1992; Breen, 1992).

APP knockout mice show no apparent abnormalities (Zheng et al., 1996), which can probably be attributed to the substitution of APP functions by homologous APP-like proteins APLP1 and APLP2 (Heber et al., 2000). However, double knockouts of APP and APLP2 show early lethality (Von Koch et al., 1997; Heber et al., 2000) while double knockouts of APP and the nervous system-specific APLP1 display no apparent abnormalities (Heber et al., 2000). Consequently, the putative functional overlap of APP and APLPs precludes an assessment of the contribution of only APP in these knockout mice.

Similarly, the roles of A β , which is also produced under physiological conditions, are obscure. Recently, a role in neuronal activity has been proposed by Kamenetz and coworkers, who showed that A β is formed in response to neuronal activity, inhibiting further excitation of surrounding neurons in a kind of feedback mechanism (Kamenetz et al., 2003; Esteban, 2004).

1.3.3.1.2 APP transgenic mice

Various transgenic mouse lines have been generated in recent years in an attempt to mimic AD pathology in brain tissue, among these mice expressing full-length APP, the C-terminal fragment or only the A β sequence [reviewed in (Seabrook and Rosahl, 1999; Hock, Jr. and Lamb, 2001)]. Despite differences in promoters, genetic background,

number and kind of APP mutations and amino acid isoform of APP expressed, most of these transgenic mice consistently show an age-dependent accumulation of A β . Also formation of A β plaques, similar to the plaques detected in human AD patients, could be mimicked in some transgenic models. However, plaques could not be detected in models where A β formation is very low (Czech et al., 1994; Malherbe et al., 1996). Obviously, the relatively short life span of mice with approximately 24 to 30 months on average is not sufficiently long to result in detectable formation of A β plaques when A β levels are very low. Albeit A β plaques in transgenic mice are somewhat different from the plaques in human AD brains (Kuo et al., 2001; Kalback et al., 2002), APP transgenic mice nevertheless represent one of the best animal models to study pathogenic events relevant to AD.

It must be mentioned, however, that – despite the presence of plaques in brains from APP transgenic mice and detectable hyperphosphorylated tau protein (Sturchler-Pierrat et al., 1997; Moechars et al., 1999a) – the formation of neurofibrillary tangles could not be reproduced in single APP transgenic mice. Tangle formation could only be detected in mice double transgenic for mutant APP and a mutant form of human tau (Lewis et al., 2001). Of note, tangle formation was accelerated by the presence of mutant APP in these mice, and similarly tangle formation could be accelerated by injection of A β ₁₋₄₂ fibrils into the brains of mutant tau transgenic mice (Götz et al., 2001), suggesting a primary role for A β and/or APP in the initiation of tau phosphorylation and tangle formation.

Expression of mutant APP in cell culture models induces cell death (Yamatsuji et al., 1996; Zhao et al., 1997) or sensitizes towards apoptotic stimuli (Eckert et al., 2001c). Similarly, neuronal death has been detected in brains of transgenic mice expressing A β , the C-terminal fragment or full-length APP (LaFerla et al., 1995; Oster-Granite et al., 1996; Calhoun et al., 1998; Moechars et al., 1999b; Urbanc et al., 2002). Although these results are controversial (Irizarry et al., 1997; Takeuchi et al., 2000), APP transgenic mice exhibit cognitive deficits (Gordon et al., 2001; Chishti et al., 2001; Higgins and Jacobsen, 2003; Van Dam et al., 2003), which have been detected also in the absence of plaques, suggesting that plaque-independent mechanisms of APP toxicity cause cognitive impairment. Neurotoxic mechanisms triggered by overexpression of mutant APP or A β accumulation have been intensively studied and several explanatory hypotheses have been proposed, among these also oxidative toxicity of A β (see 1.3.4.2).

1.3.3.2 Presenilins

The presenilins (PS) 1 and 2 are integral membrane proteins with probably 8 transmembraneous domains (Li and Greenwald, 1998), although other conformations with 7 or up to 10 transmembrane domains have also been suggested (Doan et al., 1996; De Strooper et al., 1997; Lehmann et al., 1997; Dewji and Singer, 1997b). PS1 and PS2 show 80 % amino acid homology. They are widely expressed throughout the body in the CNS as well as the periphery. PS1 and PS2 show similar distribution in the brain, where they are mainly localized to neurons and are subcellularly located mainly in the ER and Golgi complex (Kovacs et al., 1996; Walter et al., 1996), but have also been identified on the nuclear membrane (Li et al., 1997) and at the cell surface (Dewji and Singer, 1997a). Presenilins are posttranslationally modified by phosphorylation and, most prominently, by autoproteolytical cleavage yielding an approximately 30 kDa N-terminal fragment and an approximately 20 kDa C-terminal fragment (Thinakaran et al., 1996; Podlisny et al., 1997) so that only low amounts of full-length presenilin can be detected in brain tissue (Podlisny et al., 1997) (see Figure 1.7).

So far, more than 100 different mutations in the PS1 gene have been identified that account for the majority of familial early-onset cases of AD [reviewed in (Lleo et al., 2004)]. Some mutations produce phenotypes with a very early onset – even patients with an age of onset before the third decade of life were described (Campion et al., 1995; Dowjat et al., 2004). In contrast, only few mutations have been identified in the PS2 gene. PS mutations are always single amino acid mutations with the exception of one deletion mutant identified in PS1. The mutations leading to FAD identified so far are spread accross the whole length of the proteins – unlike the FAD APP mutations which are in or close to the A β sequence – and are mostly located in the hydrophobic transmembrane regions, suggesting some kind of altered membrane insertion properties of presenilins in pathogenic events causing AD. However, some mutations have also been detected in the extramembraneous loops.

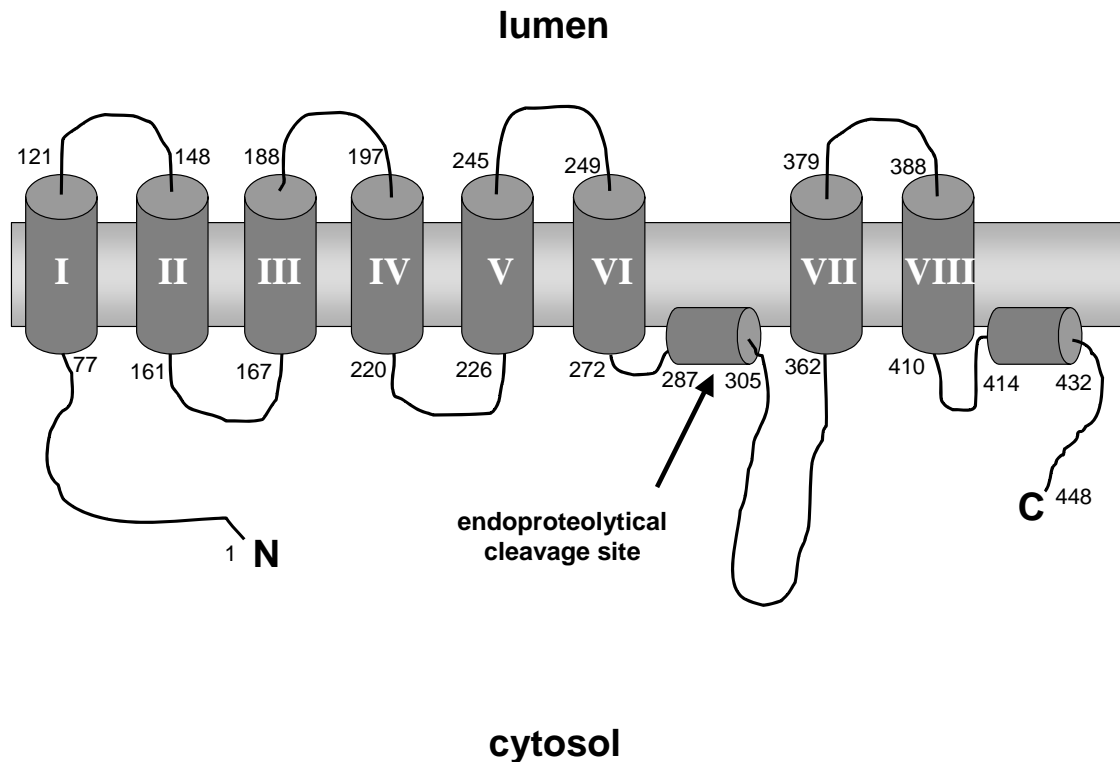


Figure 1.7: Membrane topology of presenilin 1.

Presenilin 1 contains 10 hydrophobic domains which are either transmembrane domains or membrane-associated. A topology with 8 transmembrane regions has been suggested by Li and Greenwald (Li and Greenwald, 1998). Presenilins are proteolytically processed within the large cytosolic loop resulting in formation of N-terminal and C-terminal fragments.

The discovery that mutations in the presenilins PS1 (Rogaev et al., 1995) or PS2 (Sherrington et al., 1995) – similar to APP mutations – lead to elevated formation of A β_{1-42} in cell culture studies as well as in plasma and brains of FAD patients (Duff et al., 1996; Scheuner et al., 1996; Borchelt et al., 1996; Lemere et al., 1996; Citron et al., 1997) gave further support to the amyloid cascade hypothesis. Wolfe and associates initially suggested that presenilins are directly responsible for γ -secretase activity and provided evidence that aspartate residues in each the N- as well as the C-terminal fragments are required for γ -secretase activity in a kind of aspartyl-protease manner (Wolfe et al., 1999). Later, other cofactors Nicastrin, aph-1 and pen-2 were identified that interact with presenilins in the γ -secretase complex (Yu et al., 2000; Kimberly et al., 2003).

1.3.3.2.1 Putative physiological roles for the presenilins

Apart from their putative catalytic role in the γ -secretase complex, presenilins seem to exert several functions in cell signaling and survival which are not yet completely understood. Presenilins have been shown to directly interact with APP (Weidemann et al., 1997; Waragai et al., 1997; Xia et al., 1997) and APLP (Naruse et al., 1998). They have also been suggested to regulate APP processing via modulation of intracellular APP trafficking (Naruse et al., 1998). Furthermore, presenilins interact with several proteins: with Notch (Wong et al., 1997; Struhl and Greenwald, 1999; De Strooper et al., 1999), catenins (Yu et al., 1998; Murayama et al., 1998), with the neuronal calcium-binding protein calsenilin (Buxbaum et al., 1998a), with G₀-proteins (Smine et al., 1998), with μ -calpain (Shinozaki et al., 1998) and with components of the cytoskeleton (Zhang et al., 1998; Sych et al., 2000). PS1 can furthermore directly bind tau protein and glycogen synthase kinase GSK-3 β , which is thought to be involved in the hyperphosphorylation of tau protein leading to neurofibrillary tangle formation in AD brains. PS1 mutations increase this interaction (Takashima et al., 1998) suggesting a role for presenilins in abnormal tau phosphorylation in AD brains.

The generation of presenilin knockout mice was hindered by the fact that the homozygous knockout is lethal at early stages. Embryos of presenilin 1 knockout mice show abnormal skeletal deformations and early death before or shortly after birth (Shen et al., 1997; Qian et al., 1998). This effect is even more pronounced when PS1 and PS2 are both knocked out (Herreman et al., 1999), which may be related to deficient Notch signaling, since presenilins have been shown to process Notch (De Strooper et al., 1999) and Notch knockout mice exhibit similar pathologies as PS knockout mice (Conlon et al., 1995; Wong et al., 1997).

Recent advances in the analysis of effects of a presenilin knockout in adult animals have been made by generation of mice where the PS1 gene is selectively knocked out in adult mice (Yu et al., 2001). These mice show only subtle cognitive deficits. Mice transgenic for APP and with a knockout of PS1 show greatly reduced A β accumulation and restored long-term potentiation compared to APP transgenics. Cognitive deficits induced by the APP transgene were however not reversed by the PS1 knockout (Dewachter et al., 2002). These studies imply a role of presenilins in long-term potentiation, but further studies on these mouse models are needed to broaden the knowledge on physiological roles of the presenilins in cell biology.

Studies on cell culture models with overexpression of presenilins have produced conflicting results. Proapoptotic effects have been observed for wildtype PS1 (Wolozin et al., 1998) and wildtype PS2 (Wolozin et al., 1996; Deng et al., 1996; Janicki and Monteiro, 1997), for mutant PS1 (Guo et al., 1997; Wolozin et al., 1998; Bursztajn et al., 1998; Weihl et al., 1999), and mutant PS2 (Janicki and Monteiro, 1997). However, antiapoptotic effects of wildtype PS1 (Bursztajn et al., 1998) have also been reported. Furthermore, no proapoptotic effects of wildtype or mutant PS2 have been observed in a recent study in different cell types (Gamliel et al., 2003). These contradictory results could be due to different levels of PS overexpression, as it was recently reported that low expression of wildtype PS1 exhibited no proapoptotic effect, whereas high expression of wildtype PS1 did (Hashimoto et al., 2002). Similarly, in primary cultured neurons of transgenic rats expressing wildtype PS1, increased apoptosis correlated with the amount of PS1 expression (Czech et al., 1998).

1.3.3.2.2 PS transgenic mice

Several PS transgenic mouse models expressing human wildtype and/or mutant PS1 or PS2 have been developed so far (Duff et al., 1996; Borchelt et al., 1996; Citron et al., 1997; Oyama et al., 1998; Qian et al., 1998; Davis et al., 1998; Leutner et al., 2000), but none of these mouse models exhibit A β plaque formation in brain parenchyma, despite increased formation of endogenous rodent A β . This can probably be attributed to subtle differences in three amino acids in rodent A β compared to the human A β sequence, which confers a reduced tendency towards aggregation to the rodent peptide (Dyrks et al., 1993).

Despite the absence of rodent A β plaques, mutant PS1 transgenic mice show accelerated neurodegeneration (Chui et al., 1999; Sadowski et al., 2004), suggesting that a proapoptotic role of mutant PS as identified in cell culture expression systems may be of relevance in animal models as well. Furthermore, PS transgenic mice exhibit altered long-term potentiation (Parent et al., 1999; Schneider et al., 2001b) and dysregulated calcium homeostasis (Begley et al., 1999; Yoo et al., 2000; Leissring et al., 2000; Eckert et al., 2001b; Schneider et al., 2001b). Similar effects have been observed in studies in peripheral cells from FAD patients bearing PS mutations (Gibson et al., 1996), suggesting that the effects of PS mutations in cell culture and animal models may play a direct role in the pathology of FAD in humans.

Expression of both mutant APP and mutant PS in transgenic mice results in

dramatically accelerated plaque formation in the brains of these mice (Borchelt et al., 1997; Citron et al., 1997; Holcomb et al., 1998), which is consistent with the effect of PS mutations on enhanced γ -secretase cleavage of APP. Furthermore, a triple transgenic mouse model of AD coexpressing mutant human APP, mutant PS1 and mutant tau has been recently described (Oddo et al., 2003). These mice show synaptic dysfunction and deficits in long-term potentiation, followed by A β plaque deposition and subsequent neurofibrillary tangle formation (Oddo et al., 2003), thereby closely mimicking AD neuropathology. Since A β deposition precedes the formation of tangles, a pathogenic role for A β species in eliciting tau pathology consistent with the amyloid cascade hypothesis has been suggested.

1.3.4 A role for oxidative stress in AD?

The pathogenic events that lead to the neurodegeneration observed in the brains of AD patients have not been discovered so far. Immunohistochemical studies of post mortem AD brains have established that neurons undergo apoptotic cell death (Su et al., 1994; Smale et al., 1995). Since reactive oxygen species can elicit apoptotic signaling (see 1.1.3.5), the hypothesis that oxidative stress is involved in the pathogenic steps that lead to the development of AD has been proposed in the 1990s by several groups (Blass and Gibson, 1991; Benzi and Moretti, 1995; Smith et al., 1995; Markesbery, 1997).

1.3.4.1 Oxidative stress in sporadic AD patients

Oxidative stress has been associated with the risk factors for sporadic AD, most prominently with aging (see 1.2.3). Consequently, oxidative stress markers have also been analyzed in AD patients. However, some conflicting results have been reported, furthermore, some studies analyzed only singular aspects of oxidative toxicity which makes a comprehensive overall assessment of the contribution of oxidative stress to AD pathology difficult.

Increased markers of oxidative stress, such as lipid peroxidation products, oxidatively modified proteins and nucleic acids, have been identified in sporadic AD patients [reviewed in (Christen, 2000; Pratico and Delanty, 2000; Gibson and Huang, 2002)]. Furthermore, samples from AD brains display a higher susceptibility towards *in vitro* oxidation (Hajimohammadreza and Brammer, 1990; McIntosh et al., 1997; Schippling et al., 2000). However, results are controversial as unchanged levels of lipid peroxidation products in brains from AD patients have also been reported

(Hajimohammadreza and Brammer, 1990; Hayn et al., 1996; Lyras et al., 1997; McIntosh et al., 1997).

Since increased oxidative damage can be the result of either increased production or insufficient detoxification of ROS, both mechanisms have been studied, again with conflicting results. Deficiencies in mitochondrial proteins that could contribute to increased ROS formation have been detected in brains from AD patients (Parker, Jr. et al., 1990; Parker, Jr. et al., 1994; Mutisya et al., 1994; Fukuyama et al., 1996; Chandrasekaran et al., 1997; Aksenov et al., 1999; Kish et al., 1999; Maurer et al., 2000). Furthermore, markers of inflammatory reactions have been detected in AD brains [reviewed in (Akiyama et al., 2000)] which could exacerbate oxidative stress toxicity. Other prooxidative factors identified in AD brains are increased monoamine oxidase B activity (Gottfries et al., 1983; Reinikainen et al., 1988; Kennedy et al., 2003) and increased levels of potentially prooxidative heavy metals like iron (Deibel et al., 1996; Cornett et al., 1998).

Antioxidant parameters in AD brains have been reported to be either increased (Lovell et al., 1995; Bruce et al., 1997; Ramassamy et al., 1999; Karelson et al., 2001), decreased (Marcus et al., 1998; Omar et al., 1999) or unchanged (Hayn et al., 1996). Moreover, the analysis of antioxidant parameters alone seemingly does not allow for a conclusion with regard to oxidative damage, since increased lipid peroxidation products have been identified in AD patients accompanied by either increased (Lovell et al., 1995) or decreased (Marcus et al., 1998) antioxidant enzyme activities.

In conclusion, although the majority of available experimental evidence supports the hypothesis that oxidative stress is involved in the pathogenesis of AD, conflicting results have been reported. Furthermore, it remains to be elucidated whether oxidative stress occurs early in the disease or is just a consequence of disease progression.

1.3.4.2 Oxidative stress and A β toxicity

Since the proposal of the amyloid hypothesis of AD, toxic mechanisms of A β have been extensively studied. Cells exposed to A β undergo apoptotic (Loo et al., 1993; Forloni et al., 1993) or necrotic (Behl et al., 1994a) cell death, although relatively high concentrations are needed to elicit necrosis. The toxicity of A β has been shown to be mediated by ROS (Behl et al., 1994b; Mark et al., 1997). Furthermore, toxicity of A β depends on its aggregation state (Pike et al., 1991a), which can be influenced by oxidation: oxidized A β peptides tend to aggregate more quickly (Dyrks et al., 1992).

Thus, oxidative stress can cause formation of toxic A β species, which in turn can further exacerbate accumulation of ROS.

Toxicity of A β has also been evidenced in animal models of the disease. Injection of fibrillar A β into the brains of rats leads to increased expression of iNOS in glial cells (Weldon et al., 1998). Furthermore, immunohistochemical studies have found increased markers of oxidative stress in brains of transgenic mice bearing APP mutations (Pappolla et al., 1998; Smith et al., 1998). Also, activated glial cells surrounding the plaques in transgenic mice and increased markers of inflammation have been identified (Matsuoka et al., 2001b). Since inflammatory reactions are linked to increased ROS production (see 1.1.2), a role for oxidative stress in the toxicity induced by A β seems possible. However, immunohistochemical studies do not allow for a functional analysis of the observed changes which necessitates a study of brain homogenates of transgenic mice with respect to functional oxidative stress parameters.

1.3.4.3 Oxidative stress and presenilins

Expression of presenilins in cell culture sensitizes cells to apoptotic stimuli by increasing ROS production (Guo et al., 1996), which could be reversed by antioxidants and Bcl-2 expression (Guo et al., 1997). Similarly, hippocampal neurons of PS1 transgenic mice bearing an FAD mutation showed increased sensitivity to glutamate-induced excitotoxic cell death, which was accompanied by increased ROS formation and mitochondrial damage (Guo et al., 1999b). ROS accumulation by mutant presenilins can also be closely linked to disturbed calcium homeostasis (Guo et al., 1997; Eckert et al., 2001b). Our group has previously reported changes in oxidative stress parameters in the brains of 3 months old PS1 transgenic mice (Leutner et al., 2000), which render brain tissue more susceptible to stimulated lipid peroxidation *in vivo*. However, direct evidence that basal oxidative damage is present in the brains from PS1 transgenic mice as well as a comparison of animals at different age is still lacking.

1.4 Aims of thesis

The complexity of the pathology of Alzheimer's disease AD has so far precluded any final conclusion as to the exact pathogenic steps that lead to the development of the disease. Consequently, no causal therapies for AD exist until today – necessitating the need for a better understanding of disease mechanisms for development of rational and causal future therapies. A promising hypothesis for the genesis of AD pathology lies in the contribution of oxidative stress mechanisms.

The major aim of this thesis was therefore to investigate the contribution of oxidative stress to the pathogenesis of Alzheimer's disease in several different models. By analyzing various parameters related to oxidative stress in animal models of FAD and in human postmortem brain tissue from sporadic AD patients, a comprehensive approach should be undertaken to identify causal disease mechanisms triggered by FAD mutations and sporadic AD risk factors. Furthermore, the value and relevance of transgenic mouse models compared to human AD brain tissue could be assessed.

In the first part of this thesis, the influence of aging, the most important risk factor for sporadic AD, on oxidative stress-related markers should be elucidated. To this end, markers of oxidative stress and changes in antioxidant enzymatic defense were analyzed in brain tissue during aging in C57BL/6J mice. Furthermore, age-associated changes in ROS levels were also studied in splenic lymphocytes of these mice as a peripheral cell model.

The effects of FAD mutations on oxidative stress parameters should be assessed in experiments on transgenic mice bearing human PS1 and/or APP. These mouse models have the C57BL/6J mouse strain, which was analyzed in the first part of this thesis, as genetic background. Transgenic mice were used at different age groups to elucidate the question whether aging and FAD mutations have additive effects on alterations in oxidative stress parameters. Furthermore, the transgenic mice display different formation of A β , which is a hypothetical causal agent for both the sporadic and familial forms of AD. Hence, putative correlations between various levels of A β accumulation and oxidative stress parameters could be established. Therefore, brains from transgenic mice were analyzed for lipid peroxidation products MDA and HNE as well as antioxidant enzyme activities in relation to the expression levels of human APP and A β . Furthermore, lymphocytes from PS1 transgenic mice were studied to investigate the effects of an FAD mutation on ROS levels in living cells.

In order to elucidate the influence of sporadic AD risk factors and for a comparison of the FAD-based transgenic mouse models with the much more common sporadic form of AD, post mortem brain tissue from sporadic AD patients was analyzed in the final part of this thesis. These studies should additionally allow for a correlation of risk factors for sporadic AD, like the Apo E genotype and female gender, with oxidative stress-related parameters. Furthermore, the relevance of transgenic mouse models for the study of mechanisms relevant to sporadic AD could thus be established.

In conclusion, the contribution of FAD mutations as well as risk factors for sporadic AD to putative disease mechanisms related to oxidative stress could be assessed. The results should allow for an evaluation of the relevance of oxidative mechanisms in the disease pathogenesis and provide insight whether the concept of challenging ROS formation pharmacologically could offer a rewarding approach to new therapies of AD.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Acetic acid, glacial	VWR International, Darmstadt, Germany
Acetonitrile	VWR International, Darmstadt, Germany
Agarose	VWR International, Darmstadt, Germany
Ammonium chloride, NH ₄ Cl	VWR International, Darmstadt, Germany
Bacillol®, surface disinfectant	VWR International, Darmstadt, Germany
Blue juice, 10x gel loading buffer, Gibco™	Invitrogen, Karlsruhe, Germany
BSA, Bovine serum albumine	Roche Diagnostics GmbH, Mannheim, Germany
Calcium chloride, CaCl ₂	VWR International, Darmstadt, Germany
Carbon dioxide gas, CO ₂	Messer, Griesheim, Germany
Chloroform	VWR International, Darmstadt, Germany
Complete® Protease Inhibitor Cocktail	Roche Diagnostics GmbH, Mannheim, Germany
Desoxy-d-ribose, d-Rib	Sigma-Adrich, Taufkirchen, Germany
Desoxy-nucleotide triphosphates, dNTPs	Eppendorf AD, Cologne, Germany
4,5-Diaminofluorescein-diacetate, DAF-2-DA	Calbiochem, Schwalbach, Germany
Dichlorodihydrofluorescein-diacetate, DCFH-DA	Molecular Probes, Leiden, Netherlands
Dihydroethidium, 5mM stabilized solution in DMSO	Molecular Probes, Leiden, Netherlands
Dihydrorhodamine 123, DHR	Molecular Probes, Leiden, Netherlands
Dimethylsulfoxide, DMSO	VWR International, Darmstadt, Germany
Dithiothreitol, DTT	Calbiochem, Schwalbach, Germany
DNA Ladder, 100bp	New England Biolabs, Frankfurt, Germany
ECL, enhanced chemoluminescence detection solution	Amersham Pharmacia Biotech, Freiburg, Germany
Ethanol, absolute	VWR International, Darmstadt, Germany
Ethidiumbromide, 2 % solution	VWR International, Darmstadt, Germany
Ethylenediaminetetraacetic acid, EDTA, disodium salt	VWR International, Darmstadt, Germany
FACSClean	Becton Dickonson GmbH, Heidelberg, Germany
FACSFlow	Becton Dickonson GmbH, Heidelberg, Germany
FACSRinse	Becton Dickonson GmbH, Heidelberg, Germany
Fetal calf serum, FCS	Sigma-Adrich, Taufkirchen, Germany
Forene® (INN: Isofluran)	Abbott GmbH&Co., Wiesbaden
Glutathione Reductase Assay Kit	Calbiochem, Schwalbach, Germany

Glutathione Peroxidase, Cellular, Assay Kit	Calbiochem, Schwalbach, Germany
Hank's balanced salt solution (HBSS), dry powder	Sigma-Adrich, Taufkirchen, Germany
Hydrochloric acid, 37%	VWR International, Darmstadt, Germany
Hydrochloric acid, 1 N solution	VWR International, Darmstadt, Germany
Hydrogen peroxide 3%	Sigma-Adrich, Taufkirchen, Germany
2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES)	VWR International, Darmstadt, Germany
Iron(III)chloride, FeCl ₃	VWR International, Darmstadt, Germany
Isopropanole	VWR International, Darmstadt, Germany
Kodak RPX-Omat developer	Kodak, Rochester, New York, USA
Kodak RPX-Omat fixative	Kodak, Rochester, New York, USA
Kodak Indicator Stop	Kodak, Rochester, New York, USA
Magnesium sulfate, MgSO ₄ x 7 H ₂ O	VWR International, Darmstadt, Germany
Mercaptoethanole	VWR International, Darmstadt, Germany
2-Morpholinoethanesulfonic acid, monohydrate, MES	VWR International, Darmstadt, Germany
MES running buffer NuPage	Invitrogen, Karlsruhe, Germany
Methanesulfonic acid	VWR International, Darmstadt, Germany
Methanole	VWR International, Darmstadt, Germany
1-Methyl-2-phenylindole	VWR International, Darmstadt, Germany
Milk powder, non-fat dry	Nestle Carnation, USA
Novex NuPage PreCast Gels, 4-12 % Bis-Tris, 12 wells, 1 mm thickness	Invitrogen, Karlsruhe, Germany
Novex Tris-Glycine SDS Sample Buffer 2x	Invitrogen, Karlsruhe, Germany
NuPAGE Transfer Buffer	Invitrogen, Karlsruhe, Germany
Perform	Schülke&Mayr, Norderstedt, Germany
Phenylmethylsulfonylfluoride, PMSF	Sigma-Adrich, Taufkirchen, Germany
Phosphate buffered saline, PBS, 10x concentrate	PAA Laboratories GmbH, Cölbe, Germany
Potassium hydrogen carbonate, KHCO ₃	VWR International, Darmstadt, Germany
Primers (oligonucleotides) for PCR	MWG Biotech AG
Protein marker, Magic Mark™ for protein electrophoresis	Invitrogen, Karlsruhe, Germany
Rhodamine 123, R123	Molecular Probes, Leiden, Netherlands
RPMI 1640 medium, powder	Sigma-Adrich, Taufkirchen, Germany
RPMI medium, sterile	PAA Laboratories GmbH, Cölbe, Germany
Sodium azide, NaN ₃	VWR International, Darmstadt, Germany
Sodium chloride, NaCl	VWR International, Darmstadt, Germany
Sodium dodecyl sulfate, SDS	VWR International, Darmstadt, Germany
Sodium hydrogen carbonate NaHCO ₃	VWR International, Darmstadt, Germany
Sodium hydroxide, NaOH	VWR International, Darmstadt, Germany
Sodium hydroxide, 1 N solution	VWR International, Darmstadt, Germany

Sterilium®, disinfectant for hands	VWR International, Darmstadt, Germany
Taq-polymerase (<i>Thermophilus aquaticus</i>)	Eppendorf AG, Cologne, Germany
Tween 20 (Polyoxyethylensorbitanemonolaureate)	Sigma-Adrich, Taufkirchen, Germany
Tris(hydroxymethyl)-aminomethane, Tris	VWR International, Darmstadt, Germany
Trypan Blue solution, 0.5% (w/V)	Biochrom AG, Berlin, Germany
Water, ultrapure filtered and deionized	Millipore, Eschborn, Germany

All reagents used were of analytical grade, if not otherwise specified.

2.1.2 Kits

Beta-Amyloid (A β) [1-40] colorimetric ELISA	Biosource GmbH, Solingen, Germany
BioRad DC Protein Assay Kit	BioRad, Munich, Germany
DNAEasy Tissue Kit	Quiagen, Hilden, Germany
Glutathione Peroxidase, Cellular, Assay Kit	Calbiochem, Schwalbach, Germany
Glutathione Reductase Assay Kit	Calbiochem, Schwalbach, Germany
High Pure RNA Isolation Kit	Boehringer, Mannheim, Germany
Lipid Peroxidation Assay Kit	Calbiochem, Schwalbach, Germany
Master Taq Kit	Eppendorf AG, Cologne, Germany
Superoxide Dismutase Assay Kit	Calbiochem, Schwalbach, Germany
SuperScript II Kit	Invitrogen, Karlsruhe, Germany

2.1.3 Antibodies

Primary antibodies for Western blotting:

- W0-2 antibody: monoclonal mouse anti-human APP N-terminal, amino acids 4-10 of A β sequence (Abeta GmbH, Heidelberg, Germany)
- anti-actin (I-19) sc-1616, goat polyclonal IgG, 200 μ g/ml (Santa Cruz Biotechnology Inc., Heidelberg, Germany)

Secondary antibodies for Western blotting:

- goat anti-mouse-IgG, horseradish-peroxidase conjugated, Calbiochem, Schwalbach, Germany
- rabbit anti-goat-IgG, horseradish-peroxidase conjugated, Calbiochem, Schwalbach, Germany

Antibodies for flow cytometry:

Anti-mouse CD3 (ϵ -chain), PE-conjugated, clone 145-2C11, 0.2 mg/ml,
anti-mouse CD4 (L3T4), PE-conjugated, clone RM4-5, 0.2 mg/ml,
anti-mouse CD8a (Ly-2), PE-conjugated, clone 53-6.7, 0.2 mg/ml,
rat isotype control IgG1 κ , PE conjugated, clone MOPC-31C, 0.2 mg/ml,
all purchased from Becton Dickinson, Heidelberg, Germany.

2.1.4 Buffers and media

If not otherwise stated, all buffers and media were prepared in deionized and sterile-

filtered water (Millipore). pH was adjusted with a pH-meter every day. Due to interference of Tris with the pH-meter electrode, buffers containing Tris were adjusted with the use of special pH indicator sticks ranging from pH 6.5-10.0 with 0.3 pH intervals (VWR International, Darmstadt, Germany).

2.1.4.1 Buffers used for brain tissue preparation and homogenization

EDTA solution: 1 mM NaEDTA in 0.9% NaCl, pH 7.4 at 4°C.

Tris buffer: 20 mM Tris in 0.84% NaCl, pH 7.4 at 4°C.

2.1.4.2 Buffers used for lymphocyte preparation

RPMI buffer: prepared from 1 vial of RPMI powder (10.4 g) in 1 litre of Millipore water, under addition of 10 mM HEPES (2.383 g/l) and 5% (V/V) fetal calf serum FCS. pH 7.4 at 4°C (before addition of FCS). If not otherwise stated, RPMI buffer was used for all experiments including isolation of lymphocytes, incubation of cells in 37°C waterbaths, washing steps after incubation of cells with dyes and resuspension of cells before FACS analysis.

Erythrocyte lysis buffer: 0.83 g NH_4Cl and 0.1 g KHCO_3 in 100ml Millipore water.

HBSS buffer: 1 vial of HBSS powder dissolved in 1 l Millipore water, under addition of 10 mM HEPES (2.383 g/l), 1 mM CaCl_2 and 0.5 mM MgSO_4 , pH 7.4 at 37°C. HBSS buffer was used for stimulation of cells with hydrogen peroxide, since reducing agents and proteins in serum-containing RPMI buffer lead to quenching of radicals and gave only low increases in fluorescence intensity after staining with oxidation-sensitive dyes.

RPMI buffer, sterile: 500 ml of sterile RPMI medium under addition of 10 % (V/V) FCS. Sterile RPMI buffer was used for maintenance of cells in the incubator for up to 4 hours.

2.1.4.3 Buffers for Western blots

2.1.4.3.1 Tissue lysis buffers

Tris lysis buffer: Tris 20 mM, 0.9 % NaCl, pH 7.4, plus Complete® was employed for lysis of brain tissue used for Western blotting and ELISA of soluble A β levels.

SDS solution: 2 % SDS plus Complete® was used for resuspension of the residual pellet after Tris lysis and leads to dissolution of the remaining insoluble pool of A β .

SDS lysates were employed for Western blotting for detection of insoluble A β species.

2.1.4.3.2 Buffers and solutions for SDS-PAGE, Western blot and protein detection

SDS polyacrylamide gel electrophoresis buffer: MES Running Buffer from Invitrogen® was either diluted from the commercially available 20x stock solution or prepared as follows: 0.1 M MES, 0.1 M Tris-Base, 6.93 mM SDS and 2.05 mM EDTA were dissolved in 1 l Millipore water and pH was adjusted to pH 7.3. 500 μ l of 0.5 M DTT solution was added to the inner chamber of the electrophoresis apparatus.

Blotting buffer: 50 ml of NuPAGE® Transfer Buffer 20x stock were diluted with 850 ml Millipore water, 100 ml methanol and 1 ml of 0.5 M DTT was added.

PBS: 50 ml of PBS 10x stock were diluted with 450 ml Millipore water.

TBST: A 10x concentrate was prepared from 24.2 g Tris, 80 g NaCl and 5 ml Tween in 1 l Millipore water, pH 7.6. Immediately before use, this 10x concentrate was diluted with Millipore water.

Milk: 10 % (w/V) of non fat dry milk were dissolved in TBST.

Stripping buffer: 62.5 mM Tris, 2 % SDS and 10 mM Mercaptoethanol, pH 6.0.

2.1.4.4 Buffers for DNA gel electrophoresis

TAE buffer: 4.84 g Tris, 1.142 ml acetic acid, 1mM NaEDTA in 1 l millipore water, pH 7.4.

2.1.5 Apparatus and other materials

Calibrite beads, Becton-Dickinson, Heidelberg, Germany
Centrifuge for Eppendorf caps, Microfuge R centrifuge, Beckman, Munich, Germany
Centrifuge for polypropylene tubes and microtiter plates, with buckets or microplus carriers, Centrifuge GS-6R, Beckman, Munich, Germany
Cell strainer sieve, BD Falcon™ 40 μ m, Becton-Dickinson, Heidelberg, Germany
Chromatography paper 3 mm Whatman®, Millipore, Eschborn, Germany
Combitips plus® 0.1 ml, 0.5 ml, 1 ml, 2.5 ml, 5 ml, Biopur, VWR International, Darmstadt, Germany
Coverslips for Neubauer chamber, Superior, VWR, Germany
Cryo tubes type Cryo.S, Greiner, Frickenhausen, Germany
Cuvettes, disposable, Plastibrand®, PMMA semi-micro cuvettes, Brand, supplied by VWR, Germany
Cuvettes, precision cells of special optical glass, Hellma GmbH&Co. KG, Müllheim, Germany

Ear clips for mice and compatible clamp, Hauptner, Herbolzheim, Germany
 FACSCalibur (E1034) flow cytometre, Becton-Dickinson, Heidelberg, Germany
 Fridge (4-8 °C), type Glassline KGT 3546, Liebherr-Hausgeräte GmbH, Ochsenhausen, Germany
 Freezer (-20 °C), Liebherr-Hausgeräte GmbH, Ochsenhausen, Germany
 Freezer (-80 °C), Heraeus Instruments, Hanau, Germany
 Gel electrophoresis system for DNA gels: BioRad Sub Cell Model 96 with BioRad PowerPac 300, BioRad, Munich, Germany
 Gel electrophoresis system for protein gels and Western blotting apparatus: X-Cell gel electrophoresis system, Invitrogen, Karlsruhe, Germany and BioRad Western blot transfer chamber Mini-Trans-Blot, with power supply from BioRad PowerPac 1000, BioRad, Munich, Germany
 Heating Block Unitek HB-130, CLF, Emersacker, Germany
 Hyperfilm™ ECL, Amersham Biosciences, Freiburg, Germany
 Ice machine Ziegra, Bader, Frankfurt, Germany
 Incubator Heraeus Type BB 6220, Heraeus Instruments GmbH, Hanau, Germany
 Injection needles, 20 gauge, Terumo, Leuven, Belgium
 Laminar flow hood, Heraeus Instruments GmbH, Hanau, Germany
 Magnetic agitator, type Variomag mono, H+P Labortechnik, VWR, Germany
 Microliter syringe, glass, 50µl, Hamilton, Bonaduz, Switzerland
 Microtiter plate reader, ASYS Hitech Digiscan, Eugendorf, Germany
 Microtiter plate reader, Wallac 1420 Victor²™ plate reader (Perkin Elmer, Rodgau-Jügesheim, Germany)
 Microtiter plates, 96 wells, flatt bottom, non-sterile, Greiner, Frickenhausen, Germany
 Microscope, Modell TMS, type I04, Nikon, Japan
 Millipore system for purified water Milli Q Plus PF, Millipore, Eschborn, Germany
 Multipette® plus, Eppendorf, Germany
 Neubauer chamber superior, VWR International, Darmstadt, Germany
 Nitrocellulose membrane Hybond ECL, Amersham Biosciences, Freiburg, Germany
 Parafilm® M, VWR International, Darmstadt, Germany
 PCR tubes, 0.2 ml, Greiner, Frickhausen, Germany
 pH indicator sticks, pH 6.5-10.0, VWR International, Darmstadt, Germany
 pH-meter, Multical® pH 526, Wissenschaftlich-Technische-Werkstätten WTW, Weilheim, Germany
 Photometer, Genesys 5 Spectrophotometer, Spectronic Instruments Inc., Rochester, USA
 Pipettes model Pipetman®, 20, 200, 1000µl, Abimed, Langenfeld, Germany
 Pipettes model Transferpette®, 1, 10, 100, 5000µl, Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany
 Pipette tips for 10, 100, 1000 µl and 5 ml, Greiner, Frickhausen, Germany
 Pipettes, sterile packed, for 5, 10 and 25 ml, Greiner, Frickhausen, Germany
 Pipettus®-akku, Hirschmann, VWR, Germany
 Polypropylene tubes, 15 and 50 ml, Greiner, Frickenhausen, Germany
 Polystyrene tubes Falcon 2050, Becton-Dickinson, Heidelberg, Germany
 PVDF membrane, Hybond-P 0.45µM, Amersham Biosciences, Freiburg, Germany
 Reaction tubes with cap, 1.5 ml or 2 ml, polypropylene, Greiner, Frickenhausen, Germany
 Scale type Mettler AB204 or AT 261 Delta Range®, Mettler-Toledo, Greifensee, Switzerland
 Scalpel Cutfix®, B.Braun, VWR International, Darmstadt, Germany
 Shaker IKA HS 250 basic, IKA Labortechnik, Staufen, Germany

Shaker Promax 1020, Heidolph, Kelkheim, Germany
Shaker ST6 CAT, Zipperer GmbH, Staufen, Germany
Sonifier Branson Cell Disruptor B15, Gerhard Heinemann, Schwäbisch Gmünd, Germany
Tank for liquid nitrogen Arpege 70, Air Liquide, Paris, France
Thermocycler PCR Cycler Gene Amp® PCR System 9700 PE Applied Biosciences
Tissue culture plates, polystyrene, 24 wells, flat bottom, sterile, Dunn Labortechnik GmbH, Asbach, Germany
Tissue culture plates, polystyrene, 48 wells, flat bottom, sterile, Iwaki, Dunn Labortechnik GmbH, Asbach, Germany
Tissue homogenizer, Potter S, B. Braun Biotech International, Sartorius AG, Göttingen, Germany
Transferpette®-8, 10-100 µl and 30-300 µl, Brand, Germany
Ultracentrifuge Optima™ LE-80 K with rotor 70.1 Ti, Beckman, Munich, Germany
UV transilluminator UVT-20 M/W, Herolab GmbH, Wiesloch, Germany
Video camera Variocam, PCO computer optics GmbH, Lübeck, Germany
Vortex, model REAX 2000, Heidolph, Kelkheim, Germany
Water bath Thermomix_1440, B. Braun, Melsungen, Germany
Water bath type 1003, GFL, Gesellschaft für Labortechnik mbH, Burgwedel, Germany

2.1.6 Computer software

FACSCComp software (Becton-Dickonson, Heidelberg, Germany)
Cell Quest pro software (Becton-Dickonson, Heidelberg, Germany)
Graph Pad Prism 3.0 and 4.0 (GraphPad Software Inc., San Diego, CA, USA)
Kodak ds1D software v. 2.03 (Kodak Scientific Imaging Systems, New Haven, CT, USA)
Microsoft Excel 2000 software
Mikrowin 3.0 software (Mikrotek Laborsysteme GmbH, Overath, Germany)
Wallac 1420 Workstation Version 2.00 (release 9) for Wallac 1420 Victor2™ plate reader (Perkin Elmer Life Sciences, Rodgau-Jügesheim, Germany)
Umax VistaScan software (Umax Systems GmbH, Willich, Germany)

2.1.7 Mice

Mice were housed in cages (Makrolon, type II, Altromin GmbH, Germany) with a maximum of 5 animals per cage. Each cage was supplied with a plastic tube and some sheets of cellulose. Mice were kept at room temperature and constant humidity under a 12 hour dark/light cycle with pelleted food (Standard 1320 Ratte/Maus Haltungsdiät, Altromin GmbH, Germany) and water ad libitum. Cages were replaced once weekly and cleaned with Perform disinfectant. All experiments described in this thesis are in concordance with the German law on animal care and handling of transgenic animals.

Mice with skin lesions, splenomegaly, macroscopically visible tumors, ascites or mice, that were otherwise seemingly ill, were excluded from the experiments.

2.1.7.1 C57BL/6J mice

For all experiments related to effects of aging and gender in mice, the Jackson strain of C57BL/6J mice were used. C57BL/6J@ICO mice were purchased from Iffa Credo, France. The C57BL/6J mouse was developed by Little in 1921 after crossing the female N°57 with the male N°52 from a commercial breeding centre in the USA (Miss Abby Lathrop). It was introduced into the Jackson laboratory in 1948 at the F22 generation and then to IFFA-CREDO in 1981. One infusion was done in 1986 at F159 (Jutta Davidson, Charles River Deutschland GmbH, personal communication).

2.1.7.2 Transgenic mice

The methods of generation and characterization of PS1, PDGF-APP or Thy1-APP transgenic mice have been described previously (Czech et al., 1997; Czech et al., 1998). In brief, mice were generated by pronuclei microinjection of the respective linearized DNA constructs into one of the two pronuclei of fertilized mouse embryos (C57BL/6J X SJL-F₂ hybrids, IFFA CREDO). The embryos were transplanted into the oviduct of pseudopregnant foster mothers. Transgenic offspring were detected by southern blot analysis of DNA obtained from tail samples. Southern blot analysis also showed no major rearrangement or deletion of the transgene.

All PS1, PDGF-APP or Thy1-APP transgenic mice were generated by Aventis Pharma, France, and kindly donated to our laboratory.

With the exception of APP23 mice, where frozen brain tissue obtained, all other transgenic mice for the studies in this thesis were bred in the Biocenter facilities. Female C57BL/6J mice were obtained from Iffa Credo, France, and crossed with transgenic male mice. After approximately 3 weeks pregnancy, usually 3 to 10 mice were born per litter. The offspring was genotyped as described in 2.2.7 (page 74 et seqq.) and non-transgenic littermate animals were used as controls in all experiments.

2.1.7.2.1 Presenilin-1 transgenic mice

Transgene expression of either human wildtype presenilin 1 (PS1wt) or presenilin 1 with the M146L mutation (PS1M146L) is under the control of a HMGCoA-reductase promoter. This results in a strong neuronal expression but also ubiquitous expression in peripheral tissues (Leutner et al., 2000). The M146L mutation in the presenilin sequence leads to reduced proteolytic cleavage of PS1 resulting in increased protein levels of full-length PS1 compared to PS1wt mice, which was shown in brain tissue as well as in

lymphocytes (Leutner et al., 2000). Expression of PS1wt and PS1M146L in lymphocytes was verified by RT-PCR of RNA isolated from splenic lymphocytes as described in chapter 2.2.8. PS1 mutations lead to increased amyloidogenic processing of endogenous mouse APP, however no plaques can be detected in the brains of these mice. This seems to be due to differences in certain amino acids between rodent and human A β peptide and consequently lower aggregation properties of rodent compared to human A β (Dyrks et al., 1993).

2.1.7.2.2 PDGF-APP transgenic mice

Transgenic mice bearing the 695 amino acid form of human APP with the Swedish (KM670/671NL¹), Dutch (E693Q¹) and London (V717I¹) mutations under the control of a PDGF promotor were generated as described by Blanchard and coworkers (Blanchard et al., 2003). These heterozygous PDGF-APP transgenic mice do not develop A β plaques until age of 18 months.

2.1.7.2.3 Generation of double transgenic PDGF-APP/PS1 transgenic mice

For generation of double transgenic mice, PDGF-APP transgenic male mice were crossed with and PS1M146L transgenic female mice. The offspring were genotyped as described in chapter 2.2.7, and double transgenic male mice were used for further generation of double transgenic mice. The presence of both overexpressed human mutant PS1M146L and human mutant APP in double transgenic PDGF-APP/PS1 mice leads to much higher formation of A β caused by increased amyloidogenic cleavage of APP by PS1. Consequently, PDGF-APP/PS1 double transgenic mice show accelerated plaque deposition compared to single transgenic PDGF-APP mice with first plaque formation at the age of 6 months (Blanchard et al., 2003).

2.1.7.2.4 Thy1-APP transgenic mice

The 751 amino acid form of human APP with the Swedish (KM670/671NL¹), and London (V717I¹) mutations under the control of the murine Thy1 promotor were generated as described by Blanchard and coworkers (Blanchard et al., 2003). The Thy1 promotor leads to high and selective expression in neurons. Furthermore, a Kozak element was introduced into the 5'-UTR region of the APP gene for optimization of expression. The 751 amino acid form of APP contains the 56 amino acid Kunitz

¹ Amino acid numbers refer to the amino acid numbering of APP770.

protease inhibitor (KPI) region and has been shown to result in altered APP processing with a higher yield of A β levels in cell culture (Ho et al., 1996). Consequently, A β levels are much higher in the Thy1-APP mouse model compared to PDGF-APP mice and plaque formation is accelerated with first plaques appearing already at 6 months of age (Blanchard et al., 2003).

2.1.7.2.5 APP23 transgenic mice

In cooperation with Thomas A. Bayer and Stephanie Schäfer, Homburg/Saar, Germany, brains from another APP transgenic mouse model, APP23 mice, were analysed for Cu/Zn-SOD activity. APP23 transgenic mice were generated by Novartis Pharma Inc., Basel, Switzerland, and have been first described in 1997 by Sturchler-Pierrat and coworkers (Sturchler-Pierrat et al., 1997). These mice express the 751 amino acid form of human APP with the Swedish double mutation (KM670/671NL) under the control of the murine Thy1-promotor. Plaque formation is detectable from an age of 6 months (Sturchler-Pierrat et al., 1997), and neuron loss in brains of these mice has been described (Calhoun et al., 1998). APP23 transgenic mice were housed at Novartis Pharma facilities as described in detail previously (Bayer et al., 2003), and frozen brain tissue of these mice at an age of 15 and 21 months was obtained for analysis of Cu/Zn-SOD activity.

2.1.7.3 Summary of mice used in this thesis

The following cohorts of transgenic and non-transgenic mice were used:

Non-transgenic male and female littermate controls at an age of 3-4 months, 13-15 months and 19-22 months bred in the Biocenter facilities and 3 and 25 months old female mice obtained from Iffa Credo were used for studying age- and gender-related effects on oxidative stress metabolism.

PS1wt, PS1M146L, PDGF-APP, PDGF-APP/PS1 and non-transgenic littermate controls were studied at an age of 3-4 months, 13-15 months and 19-22 months.

Thy1-APP transgenic mice and littermate controls were studied at an age of 3, 7, 12, 14 and 18 months.

Brain tissue from 15 and 21 months old APP23 transgenic mice was used for analysis of Cu/Zn-SOD activity.

2.1.8 Human brain tissue

2.1.8.1 Cohort #1

Post mortem brain tissue samples from four different brain regions (frontal cortex, parietal cortex, temporal cortex and cerebellum) from 11 Alzheimer's disease patients (7 female, 4 male) and 10 non-demented age-matched controls (6 female, 4 male) were obtained from the Brain Bank, Department of Neuropathology, Institute of Psychiatry, King's College London, United Kingdom. All brain tissue was obtained with consent of the next of kin and according to the Local Ethics Committee Guidelines of King's College London. The Alzheimer's disease patients fulfilled the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) criteria for probable AD (Mirra et al., 1991). The normal aged control brains were obtained from individuals with no history of neurological or psychiatric illness. All cases were assessed histologically using the CERAD criteria and all of the AD cases had a 'definite' diagnosis of AD (Tierney et al., 1988). The details of tissue characteristics for the different brain regions are given in Table 2.1.

Table 2.1: Characterization of brain tissue samples analyzed in cohort #1.

Brain region	Frontal cortex FC		Parietal cortex PC		Temporal cortex TC		Cerebellum Cer	
Group	Ctrl	AD	Ctrl	AD	Ctrl	AD	Ctrl	AD
total number of subjects	10	9	8	9	9	9	10	10
male	4	3	3	4	4	3	4	4
female	6	6	5	5	5	6	6	6
Age at death (years)	76.5 ± 6.9	77.6 ± 6.2	76.9 ± 8.9	78.2 ± 5.8	75.0 ± 6.9	78.8 ± 4.9	76.5 ± 6.9	77.5 ± 5.5
Post mortem interval (hours)	27.9 ± 5.8	21.9 ± 7.2	28.4 ± 7.3	19.1 ± 7.2	27.7 ± 6.6	22.3 ± 7.5	27.9 ± 5.8	20.5 ± 7.1
Apo E4 alleles								
none	6	2	5	1	5	2	6	2
heterozygous	4	3	3	3	4	2	4	3
homozygous	0	4	0	5	0	5	0	5

Ctrl = Control group, AD = Alzheimer's disease patients.

The mean age at death was not significantly different between controls and AD patients.

AD patients showed a tendency towards lower post mortem intervals in parietal cortex and cerebellum (parietal cortex $p = 0.053$, cerebellum $p = 0.084$, unpaired t-test). Furthermore, brains from AD patients had a tendency towards lower weight than control brains, which is indicative of the neurodegenerative changes and loss of brain mass associated with the disease: mean weight \pm S.E.M. was 1138 ± 46.97 g for AD patients and 1239 ± 33.74 g for controls ($p = 0.10$, t-test). Gender were distributed equally between the two groups. Among the AD patients, there were no statistically significant differences in age at death or post mortem interval among male and female patients. The major cause of death was cardiac disease in the control group and bronchopneumonia in AD patients, with no clear difference between gender. Control and patient groups differed with respect to Apo E status: among the control group, 4 subjects were heterozygous Apo E4 carriers, among the AD patients, 3 subjects (2 female, 1 male) were heterozygous and 6 subjects homozygous carriers (3 female, 3 male) (see Table 2.1). This distribution reflects the association of Apo E4 allele with an increased risk of Alzheimer's disease (Saunders et al., 1993). Due to lack of controls homozygous for Apo E4 and insufficient numbers of non-Apo E4 AD patients, statistical analysis on the effect of Apo E on antioxidant metabolism could not be performed. All tissue samples were stored at -80°C until sample preparation.

2.1.8.2 Cohort #2

In this study, only brain tissue samples from temporal cortex were used. Post mortem brain tissue samples from 16 Alzheimer's disease patients (8 female, 8 male) and 14 non-demented age-matched controls (6 female, 8 male) was provided by the Institute for Brain Aging and Dementia Tissue Repository, Irvine, California, USA. The mean age at death was 76.0 ± 2.9 and 76.8 ± 2.4 years (mean \pm 95 % C.I.) for control and AD group respectively, the mean post mortem interval was 4.87 ± 1.12 hours for controls and 5.12 ± 1.35 hours for AD patients (mean \pm 95 % C.I.) with no statistically significant differences. As already observed in samples from cohort #1, brains from AD patients had lower weight than control brains, which was statistically significant: mean weight \pm S.E.M. was 1126 ± 42.19 g for AD patients and 1299 ± 39.61 g for controls (t-test : $p < 0.01$). The major causes of death were failure of the cardiovascular system for the control group and respiratory failure for AD patients. The Apo E4 allele distribution was again inhomogenous between controls and AD patients: in the control group, 6 subjects were non-Apo E4 carriers and 3 subjects were heterozygous carriers, but no

homozygous Apo E4 control subject was available. For 5 control subjects, the Apo E4 genotype was not determined. Among the AD group, the Apo E4 allele was much more frequent: only 5 AD patients carried no E4 alleles, whereas 6 patients were heterozygous and 3 patients homozygous E4 carriers. For 2 AD patients, the Apo E genotype was not available. Of note, 3 subjects in the study carried one Apo E2 allele and these E2 carriers were all classified as controls, confirming that the Apo E2 allele exerts a protective effect against the development of AD (Corder et al., 1994; Talbot et al., 1994; Locke et al., 1995; Polvikoski et al., 1995; Hyman et al., 1996; Lippa et al., 1997). In this cohort, histological analysis of plaque load and numbers of paired helical filaments in hippocampus and frontal cortex as well as Braak stage were available for some of the brain tissue samples. Details are given in Table 2.2. AD patients had higher plaque load and number of paired helical filaments (PHF) in hippocampus as well as frontal cortex, which was not statistically significant. As would be expected, AD patients always had Braak stages of 4 to 6, while controls had Braak stages of 3 or less. All samples were stored at -80°C until sample preparation.

Table 2.2: Results of histopathological analysis of brain samples from cohort #2.

Parameter	Plaque load Frontal cortex %		Plaque load Hippo-campus %		PHFs Frontal cortex %		PHFs Hippo-campus %		BRAAK stage	
Group	Ctrl	AD	Ctrl	AD	Ctrl	AD	Ctrl	AD	Ctrl	AD
mean \pm 95% C.I.	4.67 \pm 8.03	7.17 \pm 2.78	6.28 \pm 7.92	11.0 \pm 5.40	5.34 \pm 14.9	16.8 \pm 20.8	6.96 \pm 14.1	24.9 \pm 18.9	2.33 \pm 1.44	5.50 \pm 0.88
number of subjects analyzed	5	13	8	13	5	11	8	11	3	6
p-value	0.3696 (t-test)		0.2628 (t-test)		0.4424 (t-test)		0.1308 (t-test)		p < 0.05 (Mann-Whitney-test)	

2.2 Methods

2.2.1 Preparation of tissues and cells

2.2.1.1 Preparation of murine brain tissue

Mice were killed by decapitation and brains were quickly removed. Cerebellum and brain stem were removed and the remaining cerebrum was washed once in 1 ml of EDTA-solution and twice in 1 ml of Tris buffer. The tissue was shock-frozen in cryo tubes submerged in liquid nitrogen and stored at -80°C prior to tissue homogenization.

2.2.1.2 Homogenization of human and murine brain tissue for determination of antioxidant enzyme activities and lipid peroxidation products

For determination of antioxidant enzyme activities and lipid peroxidation, human or murine brain tissue was minced in the fivefold volume of cold 20 mM Tris buffer utilizing the Potter homogenizer at 1200 rpm with 10 strokes. Human brain samples from cohort #2 were minced with 20 strokes.

An aliquot of this homogenate was diluted 1:1 with Tris buffer resulting in a 10 % (w/V) homogenate, centrifuged at 4°C and $3000 \times g$ for 10 min and supernatants were collected and stored at -80°C for lipid peroxidation assays. The remaining homogenate was centrifuged at 4°C and $8500 \times g$ for 10 min and supernatants were collected and stored at -80°C for determination of antioxidant enzyme activities.

2.2.1.3 Isolation of murine splenic lymphocytes

Mice were killed by decapitation and abdomens were sprayed with 70 % isopropanol to avoid adhesion of fur and ensure semiseptic preparation. Spleens were quickly removed and submerged in cold RPMI buffer. Spleens were cut open at one end, cells were gently smoothed out with a bent 20-gauge injection needle, dissociated with a 1000 µl pipette and passed through a 50 µm sieve. After centrifugation at $300 \times g$ for 5 min at room temperature, cells were again passed through 50 µm sieves and washed twice in RPMI buffer. For lysis of erythrocytes, cells were resuspended in 2 ml of hypotonic erythrocyte lysis buffer and kept on ice. After 2 min, lysis was stopped by adding 10 ml of RPMI buffer and washed twice with buffer. For determination of ROS with fluorescent dyes, live cells were counted via Trypan blue exclusion in a Neubauer-chamber and adjusted to 1 Mio/ml.

2.2.2 Assays of antioxidant enzymes

2.2.2.1 Superoxide dismutase assay

The assay of Cu/Zn-SOD is based on the method described by Nebot and coworkers (Nebot et al., 1993). For elimination of interfering substances and Mn-SOD-activity, Cu/Zn-SOD enzyme activity was assayed after an extraction procedure with a mixture of chloroform and ethanol (37.5 % CHCl_3 / 62.5 % ethanol). Under these conditions, Cu/Zn-SOD is resistant to denaturation, whereas MnSOD is inactivated by the solvent

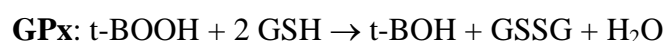
treatment. Brain homogenates were transferred to glass test-tubes and the 1.6-fold volume of CHCl_3 /ethanol mixture was added, followed by thorough vortexing and centrifugation for 10 min at $3000 \times g$ and 4°C . The clear upper layers were collected and stored on ice until assaying on the same day. For elimination of interfering mercaptanes, immediately prior to assay, samples were incubated with 1,4,6-trimethyl-2-vinylpyridinium for 1 min at 37°C . The assay is based on the photometrical measurement of the autoxidation rates of the chromophore BXT-01050 at 37°C and pH 8.8 measured in the presence V_s and absence V_c of sample. The autoxidation of BXT-01050 observed at 525 nm at alkaline pH and in oxygen-saturated assay buffer shows a sigmoidal shape with a maximum oxidation rate in a short linear range of 12 to 18 seconds. Autoxidation of BXT-01050 is accelerated by the presence of SOD activity. For each sample and controls, absorbance was measured for 2 minutes with intervals of 6 seconds. The maximum oxidation rates V_s for samples and V_c for controls with water instead of sample was calculated from the maximum changes in absorbance observed over an interval of 18 seconds. V_s was always assayed in duplicates and V_c was assayed in at least quadruplicates per test day. SOD activity was calculated from the V_s/V_c ratio of the autoxidation rates by the following equation:

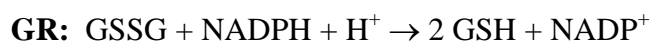
$$V_s/V_c = 1 + [\text{SOD-525 units} / (\alpha * \text{SOD-525 units}) + \beta]$$

where $\alpha = 0.073$ and $\beta = 0.93$ U. Values for α and β are empirically determined constants (Nebot et al., 1993). Activities were calculated from the above equation with Microsoft Excel software. One SOD-525 unit is defined as the activity that doubles the autoxidation background ($V_s/V_c = 2$). The commercially available Superoxide Dismutase Assay Kit from Calbiochem® was used for all experiments.

2.2.2.2 Glutathione peroxidase assay

The assay of cytosolic glutathione peroxidase GPx is based on the reaction described by Paglia and Valentine (Paglia and Valentine, 1967) using the commercially available Cellular Glutathione Peroxidase Assay Kit from Calbiochem®. Tert-butylhydroperoxide t-BOOH was used as substrate. The reduction of t-BOOH by GPx consumes reduced glutathione GSH, which is restored in a coupled enzymatic reaction of glutathione reductase GR in the presence of excess oxidized glutathione disulfide GSSG and NADPH:





The decrease in absorbance of NADPH at 340 nm can be measured photometrically.

One unit of GPx is defined as the activity that converts 2 μmol of reduced glutathione (corresponding to 1 μmol NADPH) per minute at 25°C. Enzyme activity x (expressed as [U/litre]) was calculated from the decrease in NADPH absorbance observed in an interval of 3 minutes by the following equation:

GPx activity [U/litre] = $x \mu\text{mol NADPH} / (\text{min} \times 1) = \text{change in absorbance at 340 nm per minute and 1 cm path length} [\Delta \text{Abs}/(\text{min} \times \text{cm})] / 0.00622 [(\text{Abs} \times \text{litre})/(\mu\text{mol} \times \text{cm})]$,

where 0.00622 $[(\text{Abs} \times \text{litre})/(\mu\text{mol} \times \text{cm})]$ is the extinction coefficient for NADPH at 340 nm. To determine the specific change in absorbance due to GPx activity for each sample, blank rates that control for autoxidation of NADPH by t-BOOH in the absence of sample and a control rate for unspecific oxidation of NADPH by the sample in the absence of t-BOOH were subtracted from the total sample rate. Enzyme activities were always assayed in duplicates. Activities were calculated from the above equation with Microsoft Excel software. Finally, the results were multiplied with the dilution factor in the assay and divided by the protein content determined as described in section 2.2.9.

GPx activities of human brain tissue samples from cohort #1 were measured in the Genesys 5 Spectrophotometer with 1 cm cuvette path length. All other samples were analyzed in microtiter plates in the Victor™ plate reader with a 355 nm filter with 40 nm bandpass. A correction factor of 3.026 for the unknown path length in microtiter plates filled with 240 μl assay volume compared to 1 cm path length in photometer cuvettes was determined from at least three different control enzyme samples analyzed on two separate days.

2.2.2.3 Glutathione reductase assay

Glutathione reductase activity was assayed photometrically by measuring NADPH consumption during the enzymatic reaction: in the presence of GSSG and NADPH, glutathione reductase reduces GSSG and oxidizes NADPH to yield NADP resulting in a decrease of absorbance at 340 nm (Mizuno and Ohta, 1986). We used the commercially available Glutathione Reductase Assay Kit from Calbiochem®. One unit of GR is defined as the activity that converts 1 μmol of NADPH per litre per minute at 25°C. Enzyme activity x (expressed as [U/litre]) was calculated from the decrease in NADPH absorbance observed in an interval of 5 minutes by the following equation:

GR activity [U/litre] = $x \mu\text{mol NADPH} / (\text{min} \cdot \text{litre}) = \text{change in absorbance at 340 nm per minute and 1 cm path length } [\Delta \text{Abs}/(\text{min} \cdot \text{cm})] / 0.00622 [(\text{Abs} \cdot \text{litre})/(\mu\text{mol} \cdot \text{cm})]$,

where 0.00622 $[(\text{Abs} \cdot \text{litre})/(\mu\text{mol} \cdot \text{cm})]$ is the extinction coefficient for NADPH at 340 nm.

Directly prior to the assay, brain homogenates were diluted with an appropriate amount of sample diluent to ensure a sample activity with an absorbance change in the linear range of the assay. To determine the specific change in absorbance due to GR activity for each sample, a blank rates that control for unspecific oxidation of NADPH by the sample in the absence of GSSG were subtracted from the total sample rate. Enzyme activities were always assayed in duplicates. Activities were calculated from the above equation with Microsoft Excel software. Finally, the results were multiplied with the dilution factor in the assay and divided by the protein content determined as described in section 2.2.9.

GR activities of human brain tissue samples from cohort #1 were measured in the Genesys 5 Spectrophotometer with 1 cm cuvette path length. All other samples were analyzed in microtiter plates in the Victor™ plate reader with a 355 nm filter with 40 nm bandpass. A correction factor of 2.9704 for the unknown path length in microtiter plates filled with 250 μl assay volume compared to 1 cm path length in photometer cuvettes was determined from at least three different control samples analyzed on 4 separate days.

2.2.3 Lipid peroxidation measurement

Lipid peroxidation products malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) were determined by a photometrical method utilizing the lipid peroxidation assay kit from Calbiochem® which is based on the method of Esterbauer and Cheeseman (Esterbauer and Cheeseman, 1990). The colorimetric reaction is a condensation of the respective aldehyde with 1-methyl-2-phenylindole yielding chromophores with absorption maxima at 586 nm. Both aldehydes react with 1-methyl-2-phenylindole in the presence of methanesulfonic acid and ferric iron, while substitution of methanesulfonic acid by hydrochloric acid allows measurement of MDA alone due to an irreversible cyclization reaction of hydroxyalkenals (Gerard-Monnier et al., 1998). HNE levels were calculated from absorbance levels obtained by subtracting

absorbance of samples after hydrochloric acid reaction from absorbance after methanesulfonic acid reaction. Concentrations of both aldehydes were calculated from standard curves of each aldehyde at concentrations from 0 to 20 nmol/ml. Basal levels of MDA and HNE were assayed after incubation of samples at 37°C for 30 min. Stimulated levels of MDA were assayed under the same conditions in the presence of either 50 $\mu\text{mol/l}$ FeCl_3 for mouse brain homogenates or 100 $\mu\text{mol/l}$ FeCl_3 for human brain tissue homogenates. Of note, other 2,3-unsaturated 4-hydroxy-aldehydes would give a similar reaction as HNE, but as HNE is by far the most abundant lipid peroxidation product (Mlakar and Spiteller, 1996), the resulting increase in absorbance was attributed to the presence of HNE in the samples.

2.2.4 Determination of reactive oxygen species in isolated splenic lymphocytes

2.2.4.1 Flow cytometric analysis of splenic lymphocytes

2.2.4.1.1 Basic principles of flow cytometry

Flow cytometry is a sensitive and rapid method for detection of single cells. The term derives from the measurement (“metry”) of single cells (“cyto”) that are passed through a thin capillary in solution (“flow”). This capillary is hit by a laser beam, and cells that pass the beam lead to scattering of the laser light, which is detected at two different angles: (1) the forward scatter (FSC) at low angle and (2) the sideward scatter (SSC) at 90°. The intensity of the FSC signal is related to the size of the cell: big cells lead to a higher scattering than small cells, whereas the intensity of the SSC signal is related to the morphology of the cell: cells with a lot of granules like granulocytes exhibit higher sideward scatter than cells with little cellular structure like lymphocytes. The intensities of FSC and SSC thus allow for identification of different cell populations and also for identification of intact cells, since apoptotic cells exhibit smaller size and higher granularity.

Furthermore, cells can be stained with different fluorescent dyes that are excited by the laser light and emit fluorescent light which is also detected at 90°. The optical system contains different filters which allows for simultaneous detection of several different dyes in a single cell. Figure 2.1 shows a schematic representation of the optical system in the Becton Dickinson FACSCalibur system.

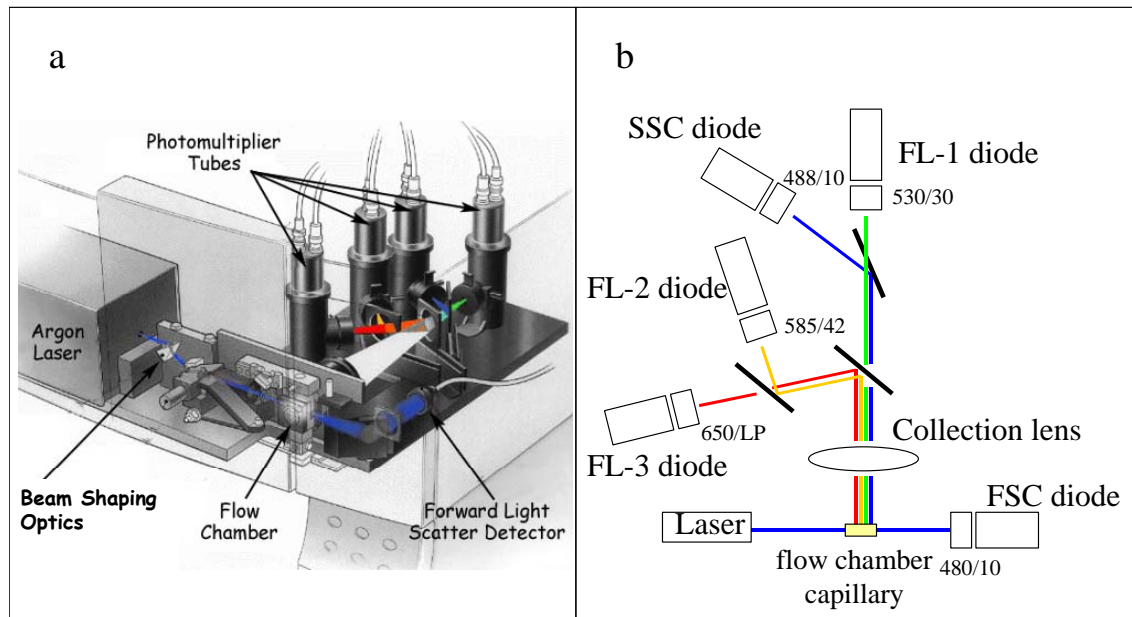


Figure 2.1: Optical system of Becton Dickinson FACSCalibur flow cytometer.

a. Graphical illustration.

b. Schematic sketch of optical paths.

The FACSCalibur system contains a 488nm blue laser, photodetectors for FSC and SSC with 488nm filters as well as photodetectors for three different fluorescence channels with filters of 530 nm (green), 585 nm (orange) and 650 nm (red). These different wavelengths are termed channels FL1, FL2 and FL3 respectively. Each single cells thus gives signals consisting of intensities in FSC, SSC and the three fluorescence channels.

Figure 2.2 depicts representative density plots (a, d) and histograms (b, c) of murine splenic lymphocytes stained with anti-CD4-PE antibody and dihydrorhodamine 123 (DHR).

Lymphocytes are cells of rather small size and granularity and thus give an FSC intensity signal between 200 and 400 and SSC intensity of around 200 (see Figure 2.2 a). Staining with anti-CD4-PE antibody leads to an increase in FL2 intensity for CD4 positive cells resulting in a peak in FL2 intensity at higher intensity between 10^2 and 10^3 , while other lymphocytes like CD8-positive cells and B-cells remain unstained and give a peak of low intensity in FL2 between 10^0 and 10^1 that represents the autofluorescence signal of cells (see Figure 2.2 b). Staining with the ROS-sensitive dye dihydrorhodamine 123 (DHR) leads to an increased signal intensity detected in FL1 between 10^2 and 10^3 (Figure 2.2 c). Since all lymphocytes are stained with DHR due to basal ROS production in living cells, no autofluorescence can be detected at low

intensities in FL1. In Figure 2.2 d, lymphocytes simultaneously stained with anti-CD4-PE antibody and DHR were analyzed by flow cytometry, and fluorescence intensities in channel FL2 (PE) was plotted versus channel FL1 (DHR). This allows for selection of double stained cells in the upper right quadrant, i.e. CD4-positiv lymphocytes stained with the ROS-sensitive dye DHR, and single stained cells in the lower right quadrant, i.e. all other lymphocytes.

Although the detectors of the different fluorescence channels are equipped with filters specific for a certain wavelength range, most fluorescent dyes show a broad emission peak and thus some light is also detected in the adjacent fluorescence channels. This overlap of emission spectra of different dyes has to be corrected by compensation if cells are simultaneously stained with several dyes. Compensation is the electronic subtraction of unwanted signal to remove the effects of spectral spillover. It allows for the specific measurement of the fluorescence intensity of the dye that is meant to be detected in the channel.

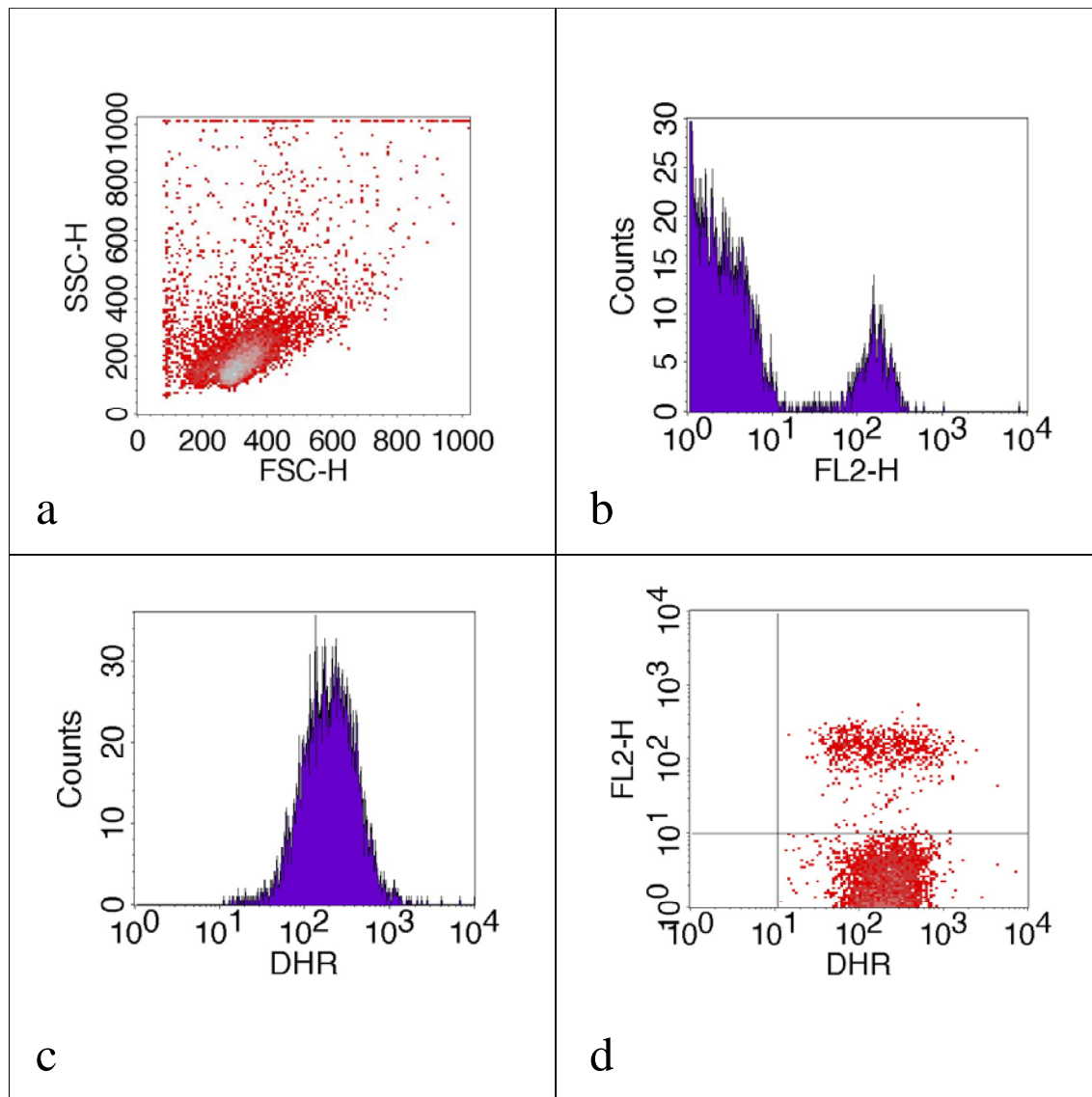


Figure 2.2: Representative density blots (a, d) and histograms (b,c) of splenic lymphocytes analyzed by flow cytometry.

a. Density plot of sideward scatter intensity (SSC-H, Y-axis) plotted vs forward scatter intensity (FSC-H, X-axis).

b. Histogram plot of FL2 fluorescence intensity of splenic lymphocytes stained with anti-CD4-PE antibody.

c. Histogram plot of FL1 fluorescence intensity of splenic lymphocytes stained with dihydrorhodamine DHR.

d. Density plots of lymphocytes double stained with DHR (X-axis) and anti-CD4-PE antibody (FL2-H, Y-axis).

See text for details.

2.2.4.1.2 Flow cytometry data analysis

For quantification of ROS in splenic lymphocytes, cells were stained with ROS-sensitive dyes and analyzed by flow cytometry using Becton Dickinson FACSCalibur flow cytometer and Cell Quest Pro software. A minimum of 8000 events were recorded per single measurement.

Lymphocytes were gated according to size and morphology in the SSC vs. FSC density plot (see Figure 2.3 a), and only the gated events, i.e. lymphocytes, were evaluated for the intensities of ROS-sensitive fluorescent dyes.

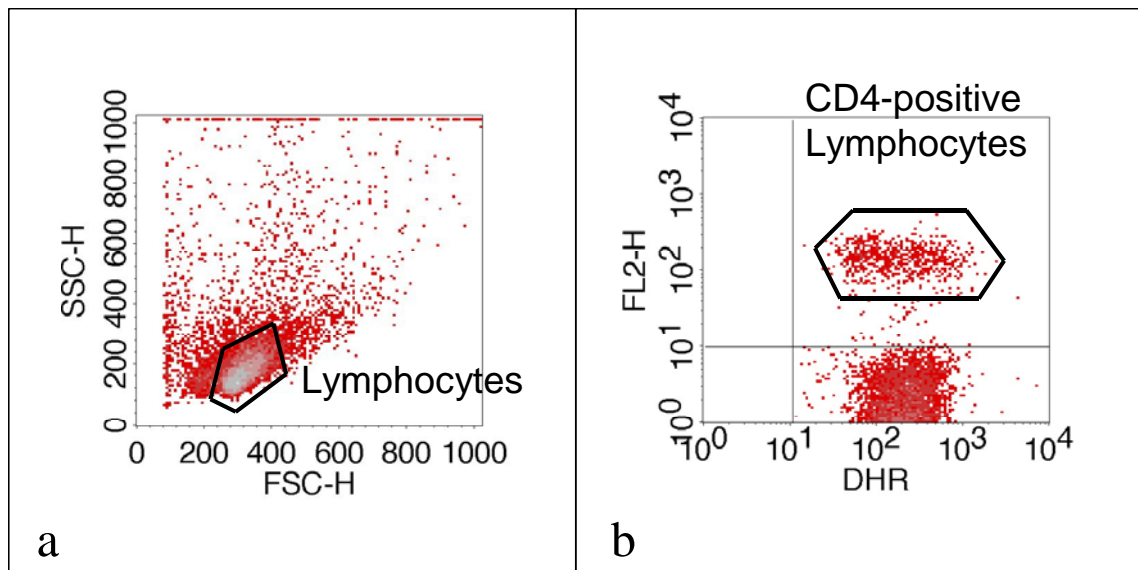


Figure 2.3: Gating strategy for lymphocytes or lymphocyte subpopulations.

a. Gating of lymphocytes from the SSC vs. FSC density plot.

b. Gating of lymphocyte subpopulations from density plots after double staining with ROS-sensitive dyes (DHR, X-axis) and PE-conjugated antibodies, e.g. anti-CD4-PE antibody (FL2-H, X-axis).

See text for details.

For quantification of mitochondrial ROS in lymphocyte subgroups, cells were double stained with dihydrorhodamine 123 (DHR) and anti-CD3-, anti-CD4- or anti-CD8-PE antibodies, and double stained cells were gated from the density plots of FL1 (DHR) vs FL2 (PE) in the upper right quadrant (see Figure 2.3 b).

For quantification of ROS, a histogram plot of gated events was analyzed for the mean peak fluorescence intensity of the respective ROS-sensitive dye. A threshold of more than 10¹ was applied to histogram analysis, since intensity values of less than 10¹ represent only the inherent autofluorescence signals of cells even in the absence of fluorescent dyes.

2.2.4.2 Staining of lymphocytes with ROS-sensitive fluorescent dyes

In general, 1 Mio/ml splenic lymphocytes were incubated with ROS-sensitive dyes at 37°C, washed twice and resuspended for FACS analysis. Cells were analyzed immediately after staining and always kept on ice in the dark until measurement.

If lymphocyte subsets were costained with PE-conjugated anti-CD3-, anti-CD4- or anti-

CD8-antibodies and IgG isotype control antibody, cells were incubated with DHR, washed, incubated with 2 µl/ml antibody solution for 15 min on ice, washed again and resuspended for FACS analysis. Phycoerythrin PE is a 240 kDa protein containing 34 phycoerythrobilin fluorochromes per molecule resulting in high fluorescence intensities. When excited by 488 nm laser light it shows an emission maximum at 564 nm and can be detected in FL2.

Basal levels of ROS were assayed immediately after lymphocyte isolation in RPMI medium. Simulation of ROS formation was assayed after transferring lymphocytes to HBSS buffer and incubation with hydrogen peroxide at 1, 10 or 100 µM for 30 min at 37°C. After stimulation, cells were centrifuged, resuspended in RPMI medium and stained with ROS-sensitive dyes as described above.

Table 2.3 gives details on the fluorescence excitation and emission maximum wavelengths, concentrations, incubation times and ROS-specificity of dyes used for determination of various ROS in splenic lymphocytes. All dyes show an excitation maximum close to 488 nm and can thus be easily excited with the laser in the FACSCalibur.

Table 2.3: Properties and staining parameters of ROS-sensitive dyes used in lymphocyte studies.

Dye	max. Exc (nm)	max. Em (nm)	FACS detection channel	Concentration	Incubation time at 37°C	Dye specificity
Dihydro-rhodamine 123 (DHR)	507	529	FL1	10 µM	15 min	peroxides, hydroxyl radical, mitochondrial ROS, peroxynitrite
Rhodamine 123 (R123)	507	529	FL1	1 µM	15 min	mitochondrial membrane potential, substrate of P-glycoprotein
Dichlorodihydro-fluorescein-diacetate (DCFH-DA)	504	525	FL1	10 µM	30 min	peroxides, cytosolic ROS, peroxynitrite
Dihydroethidium (DHE)	523	604	FL2	5 µM	30 min	superoxide
Diamino-fluorescein-2-diacetate (DAF-2-DA)	495	515	FL1	2.5 µM	30 min	nitric oxide

Emission and excitation maximum wavelengths, detection channels, concentrations and incubation times as well as ROS specificity of fluorescent dyes used for studies of ROS in murine splenic lymphocytes.

Dihydrorhodamine 123 (DHR) is the non-fluorescent reduced form of rhodamine 123 (R123). Due to its lipophilicity it can easily diffuse through cell membranes. Inside the cell, it is oxidized by various ROS to the positively charged fluorescent rhodamine R123 which is incorporated into mitochondria in a manner dependent on mitochondrial membrane potential (Johnson et al., 1980). Although several ROS could be responsible for oxidation of DHR (Hempel et al., 1999), it has been described to be most sensitive towards oxidation by hydrogen peroxide (Walrand et al., 2003), especially in the presence of cytochrome c oxidase in mitochondria (Royall and Ischiropoulos, 1993) and can thus be used as a marker for mitochondrial ROS production. The emission maximum of R123 is at 529 nm and can be detected in channel FL1.

Since the fluorescence signal obtained after staining of lymphocytes with DHR depends on two parameters, i.e. (1) oxidation of the dye and (2) incorporation into mitochondria, lymphocytes were also stained with the oxidized form of DHR, R123, to correct for possible mitochondrial defects that would affect uptake of oxidized DHR. R123 is widely used in several cell models as an indicator of mitochondrial membrane potential (Johnson et al., 1980). In lymphocytes, however, uptake of R123 into mitochondria is not useful for measurement of mitochondrial membrane potential, but rather a marker for lymphocyte activation accompanied by increased mitochondrial mass and activity (Nairn et al., 1979; Ferlini et al., 1995). Furthermore, R123 is a substrate for P-glycoprotein (Efferth et al., 1989), which is expressed in murine lymphocytes and affects R123 staining of cells (Bommhardt et al., 1994).

Dichlorodihydrofluorescein-diacetate (DCFH-DA) is a widely used ROS-sensitive dye. In its diacetate form, it readily diffuses across cell membranes, but it is converted to the more hydrophilic alcohol dichlorodihydrofluorescein (DCFH) by esterases in the cytosol and retained in the cell (Bass et al., 1983; Rothe and Valet, 1990). However, despite the higher polarity of DCFH, some dye still leaks from the cells. Therefore, FACS measurements were completed within 30 minutes after staining. DCFH is oxidized by various ROS to the fluorescent dichlorofluorescein DCF (Hempel et al., 1999). Mainly hydrogen peroxide in combination with enzymatic peroxidase activity seems to be responsible for DCFH oxidation (Walrand et al., 2003). DCFH does not stain mitochondria (Diaz et al., 2003), but rather seems to be oxidized by ROS in the cytosol. Thus, it is a useful tool for monitoring cytosolic ROS levels. The emission maximum of DCF is at 525 nm and can be detected in channel FL1.

Both DHR and DCFH have been described to be also oxidized by peroxynitrite (Crow, 1997). Peroxynitrite is formed in a reaction of the superoxide radical anion with nitric oxide (Huie and Padmaja, 1993). In order to assess a possible contribution of peroxynitrite to DHR and DCFH oxidation in our studies, we also monitored superoxide and nitric oxide levels.

Superoxide anions are readily detected by oxidation of dihydroethidium (DHE) (Rothe and Valet, 1990). Upon oxidation, DHE forms ethidium that intercalates with DNA double strands in the cell. When stacked between the bases, the π -electrons of the dye interact with the π -electron systems of the DNA bases, resulting in altered fluorescence properties (Carter et al., 1994). The emission maximum of this complex is at 604 nm and can be detected in the FL2 channel.

Nitric oxide levels have been measured with daminofluorescein-2-diacetate (DAF-2-DA) (Kojima et al., 1998). In its diacetate form, DAF-2-DA is readily membrane-permeable and becomes hydrolyzed by cytosolic esterases within the cell yielding diaminofluorescein DAF. In the presence of nitric oxide, DAF is converted to a fluorescent triazole derivate that shows an emission maximum at 515 nm and is detected in FL1.

2.2.4.3 FACS analysis of splenic lymphocytes

The flow cytometer FACSCalibur was calibrated with Calibrite Beads to check that the instrument was working well. This analysis was repeated in regular intervals of 2 months.

For analysis of splenic lymphocytes, the instrument settings were determined in preliminary experiments and maintained for all other experiments. The voltages of FSC and SSC detectors were set to E00 and 443, with amplitude gains of 2.00 and 1.00 for FSC and SSC respectively. Since the ROS-sensitive dyes show different fluorescence intensities, specific instrument settings were used for each dye. Details are given in Table 2.4.

Table 2.4: Instrument settings for FACS analysis of ROS-sensitive dyes.

Dye	Voltage			Compensation (%)			
	FL1	FL2	FL3	FL1-FL2	FL2-FL1	FL2-FL3	FL3-FL2
DHR, R123	600	594	681	1	36	0	49
DCF	600	580	681	1	19	0	49
DHE	575	628	582	1	19	32.6	47.2
DAF-2-DA	600	594	681	1	0	0	49

2.2.5 Western Blot analysis of APP and A β

Mouse brain hemispheres were homogenized in 1 ml of Tris lysis buffer (2.1.4.3) containing Complete® protease inhibitor cocktail with 10 strokes at 1200 rpm in the Potter homogenizer. Homogenates were centrifuged at $15,000 \times g$ for 30 min at 4°C. Supernatants were collected and stored at -80°C until Western blotting for analysis of APP and soluble A β . The residual pellet was used for lysis in SDS solution and determination of insoluble A β .

2.2.5.1 Preparation of brain samples for determination of soluble A β

Soluble A β is defined as the proportion that remains in the supernatant after ultracentrifugation at $100,000 \times g$ for 1 hour (Kuo et al., 1996). The correct isolation of soluble A β by centrifugation at $15,000 \times g$ was tested by comparison of supernatants from $15,000 \times g$ and $100,000 \times g$ ultracentrifugations: subsequent to a 30 min $15,000 \times g$ centrifugation, aliquots of supernatants were either frozen for Western blotting or further centrifuged at $100,000 \times g$ for 1 h. The intensity of the A β band at 4 kDa is not diminished by ultracentrifugation indicating that soluble A β is already isolated by centrifugation at $15,000 \times g$ (Figure 2.4). Interestingly, the C99 band at 12 kDa is reduced after ultracentrifugation which might indicate that C99 is contained in a membrane-bound form. This would be expected since the γ -secretase complex is a membrane-bound protein complex that cuts C99 intramembraneously (Kimberly and Wolfe, 2003).

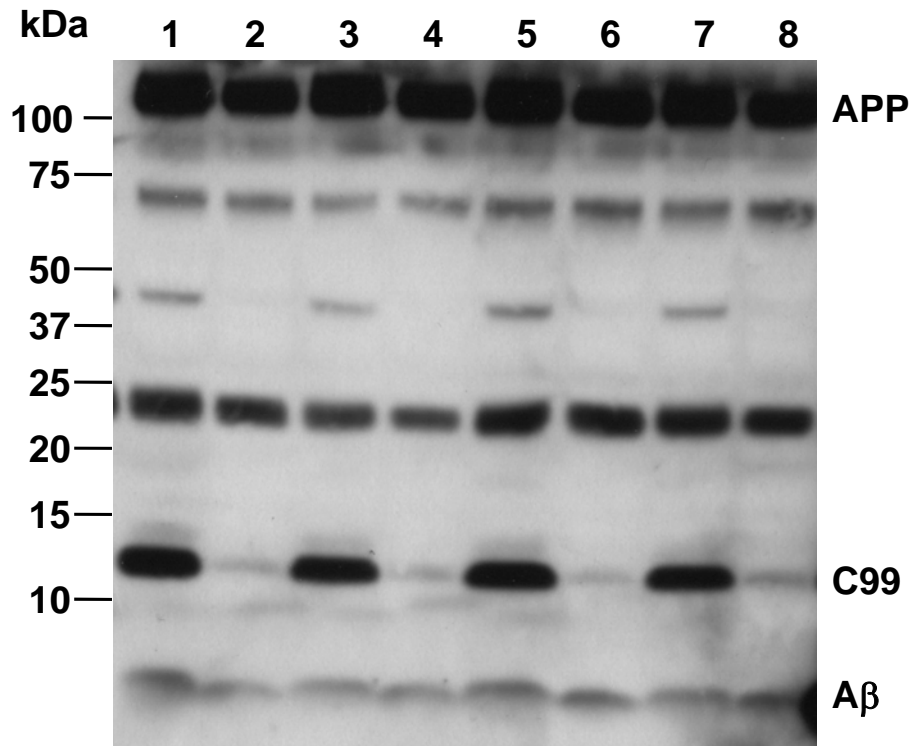


Figure 2.4: APP/A β Western blot of Tris lysates from Thy1-APP transgenic mouse brains.

Brain lysates were electrophoresed either directly after centrifugation for 30 min at $15,000 \times g$ (lanes 1, 3, 5, 7) or after a second centrifugation procedure for 1 hour at $100,000 \times g$ (lanes 2, 4, 6, 8).

2.2.5.2 Preparation of brain samples for determination of insoluble A β

Our protocol is a modification of the method first described by Kuo and coworkers (Kuo et al., 2001), who reported that insoluble amyloid beta aggregates and plaques of APP transgenic mice are soluble in buffers containing 2% SDS. After homogenization of brain hemispheres in Tris lysis buffer and centrifugation at $15,000 \times g$ for 30 min, pellets were resuspended in 500 μ l of 2 % SDS solution containing protease inhibitors (Complete®) and lysed by sonification with 20 pulses on the Branson sonifier set to level 2, 40 % output scale. Samples were kept on ice for 30 min, vortexed and centrifuged at $15,000 \times g$ and 4°C for 30 min. Supernatants were collected and stored at -80°C until Western blotting.

2.2.5.3 SDS PAGE and Western Blotting of brain extracts

Brain extracts were diluted in an appropriate amount of NOVEX 2x sample buffer containing 5% mercaptoethanol to give a protein concentration of either 1 or 2 $\mu\text{g}/\mu\text{l}$. Samples were then heated to 70°C for 10 min followed by quick centrifugation. NuPage

Bis-Tris gels with a 4-12 % concentration gradient were used for gel electrophoresis of proteins. Gels were placed in the X-Cell electrophoresis system filled with running buffer, loaded with 15 µl of diluted samples (either 15 or 30 µg of protein per lane) and electrophoresis was started at 60 Volts for approximately 5 min to ensure concentration of samples at the rim of the gel. Then, voltage was set to 200 Volts and electrophoresis continued for 30 min. Gels were removed from the gel cassettes and blotted on PVDF or nitrocellulose membranes.

2.2.5.4 Detection of APP, C99 and Abeta

After blotting, membranes were boiled in PBS for 5 min, washed in TBST and blocked with milk for 1 h at room temperature, followed by a washing step and overnight incubation at 4°C with the primary antibody W0-2 (Abeta GmbH) at a concentration of 8 µg in 15 ml TBST containing sodium azide at a concentration of 0.02 % (m/V). The next day, membranes were washed and incubated with horseradish-peroxidase-conjugated secondary antibody anti-mouse-IgG (Calbiochem) at a dilution of 1:1000 in TBST for 1 h at room temperature. After another washing procedure, membranes were exposed to ECL solution according to the suppliers instructions, bands were detected with Amersham Hyperfilm™ ECL and films were developed with Kodak solutions.

For densitometric analysis, films were scanned with Umax Astra 4000 scanner and Umax VistaScan software. Density of protein bands was evaluated with Kodak ds1D software.

2.2.5.5 Detection of actin as loading control

After detection of APP and Aβ with W0-2 antibody, blots were incubated in stripping buffer at room temperature for 1 hour, blocked in milk for 1 hour at room temperature and incubated for 1 hour with goat anti-actin antibody (Santa Cruz) at a dilution of 1:140 in 15 ml TBST containing sodium azide at a concentration of 0.02 % (m/V). Finally, membranes were incubated with secondary horseradish-peroxidase conjugated anti-goat IgG antibody (Calbiochem) 1:1000 for 1 h at room temperature. Between each incubation, blots were thoroughly washed in TBST. Detection of protein bands was performed as described above (2.2.5.4).

2.2.6 Quantification of Aβ₁₋₄₀ by ELISA

For quantification of levels of soluble Aβ₁₋₄₀, mouse brain hemispheres or human brain

tissue samples were homogenized in the fivefold volume of Tris lysis buffer (2.1.4.3) containing Complete® protease inhibitor cocktail with 10 strokes at 1200 rpm in the Potter homogenizer. Homogenates were centrifuged at $15,000 \times g$ for 30 min at 4°C. Supernatants were collected and stored at –80°C until ELISA analysis of soluble A β ₁₋₄₀.

Beta-Amyloid (A β) [1-40] colorimetric ELISA from Biosource GmbH was used for quantification of human A β ₁₋₄₀ levels. The assay specifically detects human A β ₁₋₄₀ peptides and does not cross-react with human A β ₁₋₄₂ or A β ₁₋₄₃ peptides (according to the supplier's information). Furthermore, it is specific for human vs mouse A β , since brain homogenates from non-transgenic mice containing endogenous mouse A β yielded absorption levels corresponding to 2.017 pg/ml, which is below the minimum detectable dose of 10 pg/ml.

The preparation of standard and sample dilutions and the assay procedure were conducted according to the supplier's instructions. All sample and standard dilutions contained PMSF for elimination of protease activity. The assay principle is based on a standard sandwich ELISA which utilizes precoated plates, rabbit anti-human A β ₁₋₄₀ N-terminal detection antibody and anti-rabbit IgG peroxidase-conjugated secondary antibody. Color development is started by addition of tetramethylbenzidine TMB yielding a yellow chromophore with absorbance at 450 nm. Absorbance was measured in Victor2™ plate reader (Perkin Elmer, Germany) using a 450 nm filter with 7 nm bandpass. Sample concentrations were determined from the A β ₁₋₄₀ standard curve, which was obtained from plotting the absorption of the standard dilutions versus the standard concentrations and calculation of the best-fit polynomial fourth order equation (Prism Graph Pad® Software).

2.2.7 Genotyping of transgenic mice

2.2.7.1 DNA isolation from rodent tails

DNA from rodent tails was isolated with DNAeasy tissue kit according to the supplier's manual. All steps were conducted in aseptic conditions and great care was taken to minimize possible cross-contamination between samples. Briefly, tissue samples were obtained by tail biopsy from mice weakly anaesthetized by isoflurane inhalation and mice were simultaneously marked with numbered ear clips for identification. Tail biopsy samples were lysed by addition of lysis buffer and proteinase K during an

2.2.7.2 PCR reaction

The following primers were used:

The PCR reaction gives a 425 bp product for PS1, a 324 bp or 492 bp product for the 695 or 751 amino acid form of APP, respectively, and a 311 bp product for MFH.

2.2.7.3 DNA gel electrophoresis

allowed to cool down and consolidate for 1 hour. The gels were then placed in the Sub Cell Chamber filled with an appropriate amount of TAE buffer to ensure complete submersion of gels, and the anode chamber was filled with 20 μ l ethidium bromide solution. 15 μ l of PCR reaction samples were mixed with 1 μ l Blue juice 10x gel loading buffer and loaded onto the gel. Samples were electrophoresed with Bio Rad Power Pac 300 set to 80 Volts for approximately 1 hour. Gels were then placed on a UV transilluminator (Herolab) and bands were photographed with a video camera (Variocam) and printed for analysis.

The MFH (mouse fetal hemoglobin) control yielded a band of the desired length (311 bp) for every mouse DNA sample indicating that DNA was correctly isolated. Also, PS1 and APP695 positive controls always gave bands at 425 bp and 324 bp respectively. No other bands were detected indicating specificity of primers. Negative controls with deionized water instead of DNA templates always gave negative results.

Figure 2.5 shows a representative picture of DNA gels after PCR reactions.

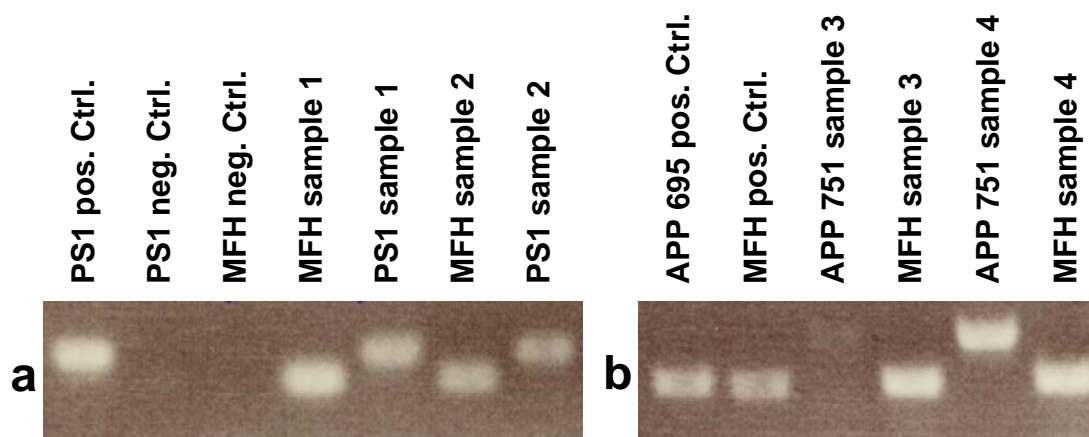


Figure 2.5: Representative DNA gel electrophoresis for genotyping of transgenic mice.

PCR reaction products of DNA samples isolated from mouse tails were electrophoresed on agarose gel stained with ethidiumbromide.

a. PS1 and MFH PCR reaction products: samples 1 and 2 PS1wt transgenic mice.

b. APP and MFH reaction products: sample 3 non-transgenic littermate, sample 4 Thy1-APP transgenic mouse.

Note the different retention of DNA bands according to the length of the PCR product: MFH 311 bp, APP695 324 bp, PS1 425 bp, APP751 492 bp.

2.2.8 RT-PCR analysis of APP and PS1 expression in splenic lymphocytes

RNA was isolated from splenic lymphocytes utilizing High Pure RNA Isolation Kit from Boehringer as described in the manufacturer's protocol. Briefly, 5 million cells were resuspended in 200 μ l PBS and lysis was started by adding 400 μ l lysis buffer.

Samples were pipetted onto columns, washed, incubated with DNase, followed by several washing steps, and finally eluted and stored at -20°C until RT-PCR.

Concentration of RNA was determined photometrically by measuring absorbance at 260 nm. The concentration was calculated from the following equation (Wilfinger et al., 1997): $1 \text{ Abs}_{260\text{nm}} = 40 \text{ } \mu\text{g} / \text{ml RNA}$.

Reverse transcriptase (RT) reactions were performed with SuperScript II Kit from Invitrogen. 64 ng of RNA were used for RT-PCR. For first strand cDNA synthesis, RNA samples were incubated with 0.5 $\mu\text{g}/\text{ml}$ oligo-dT primers and 1 mM dNTPs for 5 minutes at 65°C to ensure annealing of primers to poly-A-tails of messenger RNA. In a second step, buffer, DTT, magnesium and RNase inhibitor were added, followed by incubation at 42°C for 2 min. Then, reverse transcriptase was added and samples were incubated at 70°C for 15 minutes for reverse transcriptase reaction. In a final step, residual RNA was digested by RNase treatment at 37°C for 20 min. Reaction products were stored at -20°C until PCR. PCR of PS1 and APP DNA was performed as described above (2.2.7.2).

2.2.9 Determination of protein content

Protein content was determined according to the method of Lowry (Lowry et al., 1951). In the first step of the assay, proteins react with copper tartrate in alkaline solution. In the second step, Folin reagent¹ is added and reduced by the protein-copper-complexes resulting in a characteristic blue color due to several reduced species. The color shows an absorbance minimum at 405 nm and a maximum at 750 nm (Peterson, 1979).

The commercially available BioRad DC Protein Assay Kit was used. Briefly, 5 μl of sample dilution were mixed with 25 μl of reagent A (copper tartrate solution) and 200 μl of reagent B (Folin reagent) in a microtiter plate. If detergents were present in the samples, reagent A was supplemented with 3 % reagent S. Protein standard solutions were prepared from BSA at concentrations of 200 to 1600 $\mu\text{g}/\text{ml}$ protein in the respective sample homogenization buffer. Standards and samples were assayed in triplicates. After addition of reagents, the plate was incubated at room temperature for 15 to 30 min. Absorption was read in the ASYS Hitech Digiscan microtiter plate reader with a 620 nm filter. Protein concentration was calculated from the standard curve with Mikrowin 3.0 software (Mikrotek Laborsysteme GmbH, Overath, Germany).

¹ Folin reagent is a solution containing phospho-molybdic and phospho-tungstic acid.

2.2.10 Calculations and Statistics

For evaluation of test results, the mean of assay results per duplicate or triplicate and division by the protein content of each sample were calculated with Microsoft Excel software. The values calculated with Excel software were transferred into GraphPad Prism tables with 1 replicate per sample to calculate error bars. Thus, 1 value in the statistical analysis always represents the mean of the assay results from 1 individual sample, i.e. samples taken from one individual subject or one animal. If not otherwise stated, all data are given as mean \pm S.E.M.

If not otherwise stated, Gaussian distribution was assumed for all statistical evaluations of parametric data. For direct comparison of differences between two groups, two-tailed t-test was calculated. For comparison of three or more groups, One-way ANOVA was utilized. If two independent parameters (e.g. aging and gender) had an effect on a variable, Two-way ANOVA was calculated. Analysis of lymphocyte preparations with ROS-sensitive fluorimetric dyes was subject to high interday variations due to methodological reasons. Therefore, experiments on each individual test day were conducted in groups of samples consisting of similar numbers of each male and female controls and transgenic animals. Consequently, for statistical analysis of differences between groups in lymphocyte preparations, two-tailed paired t-test and repeated measures ANOVA were employed. ANOVA was followed by post hoc student's t-test.

Exceptions from Gaussian distributions include (i) the Braak staging in cohort #2, which represents non-parametric data, hence significance of differences between the two groups was calculated with Mann-Whitney-test, (ii) stimulated MDA levels in AD brain study cohort #2, which were distributed bimodally and analyzed by calculation of chi-squared test, and (iii) quantification of A β ₁₋₄₀ in human brain tissue, as these measurements were strongly modulated by the Apo E4 genotype and obviously not Gaussian distributed, hence differences between groups were calculated with Mann-Whitney-test.

Correlation between two different parameters was calculated assuming the data were either Gaussian distributed, giving the Pearson correlation coefficient r_P , or nonparametrically distributed, yielding the Spearman coefficient r_S . All data from biochemical assays was assumed to underly Gaussian distribution. Exceptions were made for the quantification of A β ₁₋₄₀ in human brain tissue, as these measurements were strongly modulated by the Apo E4 genotype (see Figure 3.44, page 139). Therefore, for

all correlations with $A\beta_{1-40}$ levels, a nonparametric distribution was assumed. Furthermore, the values for MMSE status, i.e. a measure of cognitive abilities, of controls and AD patients in cohort #2 were assumed to be nonparametrically distributed.

All calculations were done using Graph Pad Prism 3.0 TM software (GraphPad Software Inc., San Diego, CA, USA). Results were termed significant for $p < 0.05$.

3 RESULTS

3.1 Effects of aging and gender on ROS metabolism in brain tissue and peripheral cells of mice

3.1.1 ROS metabolism in C57BL/6J mice during aging

C57BL/6J mice were used to study several parameters related to oxidative stress during aging, since this mouse strain represents the genetic background of the Alzheimer's disease transgenic mouse models analyzed in later parts of this thesis. The average life span of C57BL/6J mice is approximately 820 days (27 months) (Kunstyr and Leuenberger, 1975; Goodrick, 1977). In order to avoid a strong influence of natural selection caused by aging, 25 months old mice were chosen as maximum age group. Accordingly, female mice of 3 to 4 different age groups were used for aging studies: young mice (3 months old), middle-aged mice (13-15 months old), aged mice (19-22 months old) and highly aged (25 months old) mice. Table 3.1 gives details on the total number of animals used for studies of oxidative stress parameters during aging.

Table 3.1: Age groups and number of mice used for aging studies.

age group	young	middle-aged	aged	highly aged*
age (months)	3-4	13-15	19-22	25*
number of animals n	29	9	11	7*

* Brains but not splenic lymphocytes of 25 months old animals were analyzed.

Brains from these mice were analyzed for levels of lipid peroxidation products malondialdehyde MDA and 4-hydroxynonenal HNE and for activities of antioxidant enzymes Cu/Zn-SOD, GPx and GR. Levels of lipid peroxidation product MDA were also studied after *in vitro* stimulation. Splenic lymphocytes were isolated for direct analysis of various reactive oxygen species in living cells with ROS-sensitive fluorescent dyes.

3.1.1.1 Oxidative damage and enzymatic antioxidant defense in murine brains during aging

In brains from C57BL/6J mice, pronounced changes in markers for oxidative stress and antioxidant defense were observed. Levels of lipid peroxidation products paradoxically decrease with aging in mouse brains, which was found for MDA (Figure 3.1 a), the more abundant product of lipid peroxidation, as well as for HNE (Figure 3.1 b), which is derived mainly from oxidation of PUFAs. While MDA levels decreased steadily with

aging, HNE levels showed some fluctuation in 19-22 months old animals. This may however be related to the higher variation of values associated with HNE measurement. Nevertheless, the mean value of 19-22 months old animals was still lower than that of 3-4 months old mice.

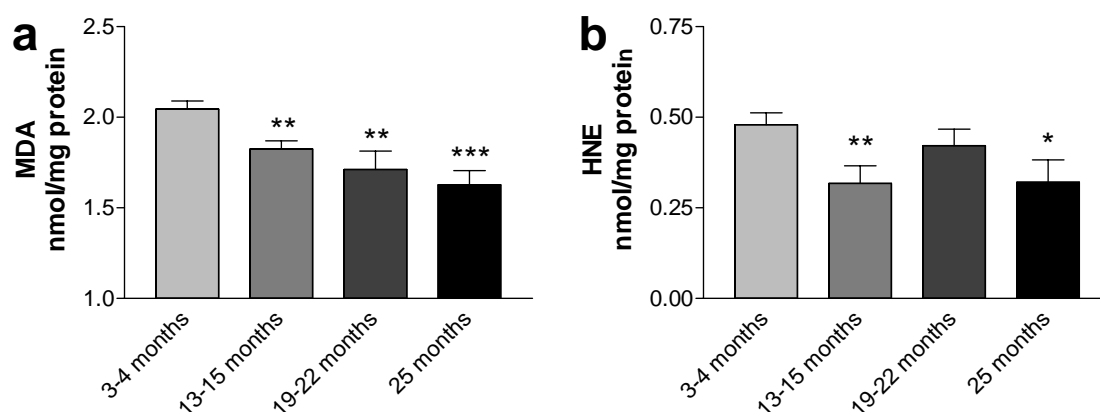


Figure 3.1: Effect of aging on levels of lipid peroxidation products in brains from C57BL/6J mice.

Lipid peroxidation products were determined in brain homogenates from young (3 months old, $n = 26$), middle-aged (13-15 months old, $n = 12$), aged (19-22 months old, $n = 11$) and highly aged mice (25 months old, $n = 7$).

a. Malondialdehyde (MDA) levels (nmol/mg protein). One-way ANOVA $p < 0.0001$. Post hoc t -test ** $p < 0.01$, *** $p < 0.001$ vs 3 months old mice.

b. 4-Hydroxynonenal (HNE) levels (nmol/mg protein). One-way ANOVA $p < 0.05$. Post hoc t -test * $p < 0.05$, ** $p < 0.01$ vs 3 months old mice.

As indirect markers of ROS metabolism, activities of several antioxidant enzymes were analyzed. These enzymes serve to detoxify ROS in subsequent reactions: Cu/Zn-dependent superoxide dismutase Cu/Zn-SOD catalyses the dismutation of superoxide radical anions, which are mainly formed in the mitochondrial respiratory chain. The Cu/Zn-SOD reaction yields molecular oxygen and hydrogen peroxide H_2O_2 , which is further decomposed either by catalase – at high concentrations of H_2O_2 – or by glutathione peroxidase GPx already at lower concentrations. Since catalase activity is low in brain, GPx activity represents the more important H_2O_2 -utilizing enzyme under physiological conditions and was therefore analyzed. The GPx reaction requires and consumes reduced glutathione as cofactor, which is regenerated by the glutathione reductase GR reaction. Therefore, the reactions of Cu/Zn-SOD, GPx and GR work in concert to detoxify ROS.

In good accordance with decreased levels of lipid peroxidation products, we found that activities of antioxidant enzymes are increased in aged mice (Figure 3.2). However, changes in enzyme activity were not uniform for the different enzymes. While activity

of GPx steadily increased with aging (Figure 3.2 b), activities of Cu/Zn-SOD and GR increased until an age of 19-22 months but declined in 25 months old animals to the levels of young mice (Figure 3.2 a and c respectively).

For analysis of ROS detoxification capacity in a functional assay, we conducted experiments of lipid peroxidation stimulation *in vitro*. Addition of ferric iron FeCl_3 to brain homogenates propagates formation of reactive oxygen species via Fenton or Haber-Weiss reactions and thus favours decomposition of lipids to aldehydes. Accordingly, increased levels of MDA can be detected in brain homogenates after *in vitro* stimulation (Figure 3.3 a). During aging, stimulation of lipid peroxidation is decreased in 13-15 months old mice compared to young mice, but increases thereafter in older mice converging to the levels of young mice (Figure 3.3 b).

These results show that antioxidant enzyme activities, rising with age, can protect against stimulation of lipid peroxidation in middle-aged mice but fail to do so in aged mice. Nevertheless, oxidative damage is not a prominent feature of aged brains since basal levels of lipid peroxidation were decreased in aged animals, which may be causally related to increased activities of antioxidant enzymes.

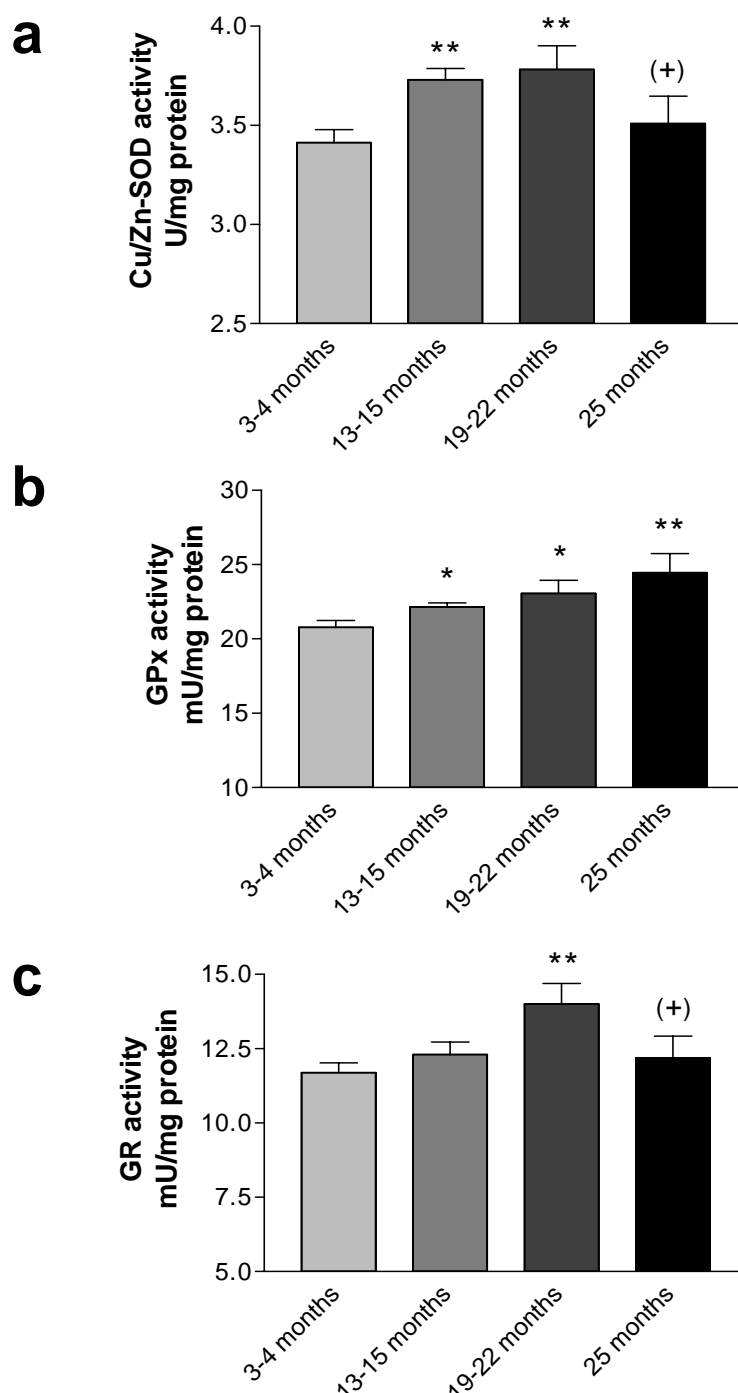


Figure 3.2: Activities of antioxidant enzymes in brains from C57BL/6J mice during aging.

Antioxidant enzyme activities were determined in brain homogenates from young (3 months old, $n = 26$), middle-aged (13-15 months old, $n = 15-16$), aged (19-22 months old, $n = 11$) and highly aged mice (25 months old, $n = 7$).

a. Activity of Cu/Zn-superoxide dismutase Cu/Zn-SOD (U/mg protein). One-way ANOVA $p < 0.01$. Post hoc t -test ** $p < 0.01$ vs 3 months old mice, (+) $p < 0.08$ vs 13-15 months old mice.

b. Activity of glutathione peroxidase GPx (mU/mg protein). One-way ANOVA $p < 0.01$. Post hoc t -test * $p < 0.05$, ** $p < 0.01$ vs 3 months old mice.

c. Activity of glutathione reductase GR (mU/mg protein). One-way ANOVA $p < 0.05$. Post hoc t -test ** $p < 0.01$ vs 3 months old mice, (+) $p = 0.1$ vs 19-22 months old mice.

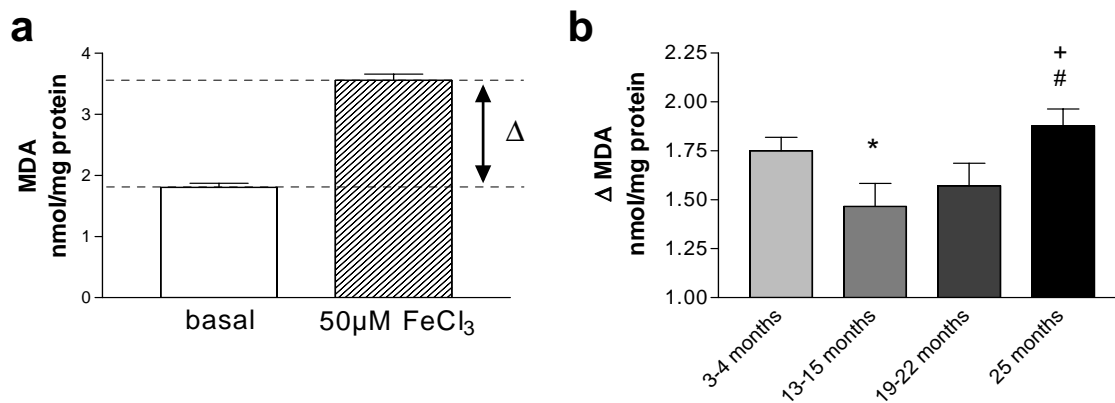


Figure 3.3: Stimulation of lipid peroxidation with FeCl₃ in young mice (a) and differences in during aging (b).

a. Effect of stimulation of lipid peroxidation with 50 μM FeCl₃ for 30 min at 37°C in brain homogenates from young mice (3 months old, n = 22).

b. Levels of stimulated MDA (nmol/mg protein) in brain homogenates from young (3 months old, n = 22), middle-aged (13-15 months old, n = 9), aged (19-22 months old, n = 8) and highly aged (25 months old, n = 6) mice. One-way ANOVA p < 0.05. Post hoc t-test * p < 0.05 vs 3 months old control mice, + p < 0.05 vs 13-15 months old mice, # p < 0.05 vs 19-22 months old mice.

3.1.1.2 ROS levels in splenic lymphocytes during aging

In addition to studying surrogate markers for oxidative stress at the levels of lipid peroxidation products in brains from aged mice, we furthermore isolated splenic lymphocytes to measure levels of reactive oxygen species (ROS) directly in living cells.

Analysis of different ROS in splenic lymphocytes showed no clear elevation with aging. Although oxidation of the mitochondrial ROS-sensitive dye DHR is increased in 13-15 months and 19-22 months old mice compared to young mice, this proved not to be statistically significant. Only a tendency towards increased ROS levels was observed in 13-15 months old mice (Figure 3.4 a). As previous studies in our group had observed a higher vulnerability of CD4-positive lymphocytes to undergo apoptosis in AD patients (Schindowski et al., 2003), we were interested if CD4-positive lymphocytes display different changes during aging compared to the whole lymphocyte population. We therefore additionally stained lymphocytes with a fluorescent anti-CD4-antibody and evaluated only double-stained cells. In the subset of CD4-positive lymphocytes similar results as in the whole lymphocyte population were observed – with highest DHR staining in 13-15 months old animals – although DHR oxidation declined significantly from 13-15 months old to 19-22 months old animals (Figure 3.4 b). The increased fluorescence of DHR in middle-aged mice is not due to better dye retention in cells as evidenced by staining with the oxidized form of DHR, R123 (Figure 3.4 c). Staining with other ROS-sensitive dyes DCFH-DA for cytosolic ROS and DHE for superoxide

radical anions showed no age-associated changes (Figure 3.4 d and e respectively). Only a prominent increase in nitric oxide levels with aging was observed (Figure 3.4 f).

Although the dyes DHR and DCFH-DA have been described to be oxidized by peroxynitrite, the observation that nitric oxide levels are elevated in aged mice but DHR and DCFH-DA oxidation is not simultaneously increased suggests that – at least under our conditions – DHR and DCFH-DA are not significantly oxidized by peroxynitrite. This is furthermore supported by the observation that oxidation of DHE, which is specifically oxidized by superoxide radical anions, is not changed during aging. Since superoxide radicals are required for formation of peroxynitrite, it seems unlikely that the increased oxidation of DHR in middle-aged animals is caused by peroxynitrite.

Overall the results of this aging study provide evidence that adaptive processes like upregulation of antioxidant enzymes take place during aging resulting in efficient detoxification of reactive oxygen species and prevention of oxidative damage in brains from mice. Moreover, the findings on brain tissue are in good accordance with the analysis of ROS in splenic lymphocytes, since in this cell type similarly no accumulation of ROS with aging was observed, suggesting that the normal physiological aging process is not inevitably accompanied by excessive ROS accumulation.

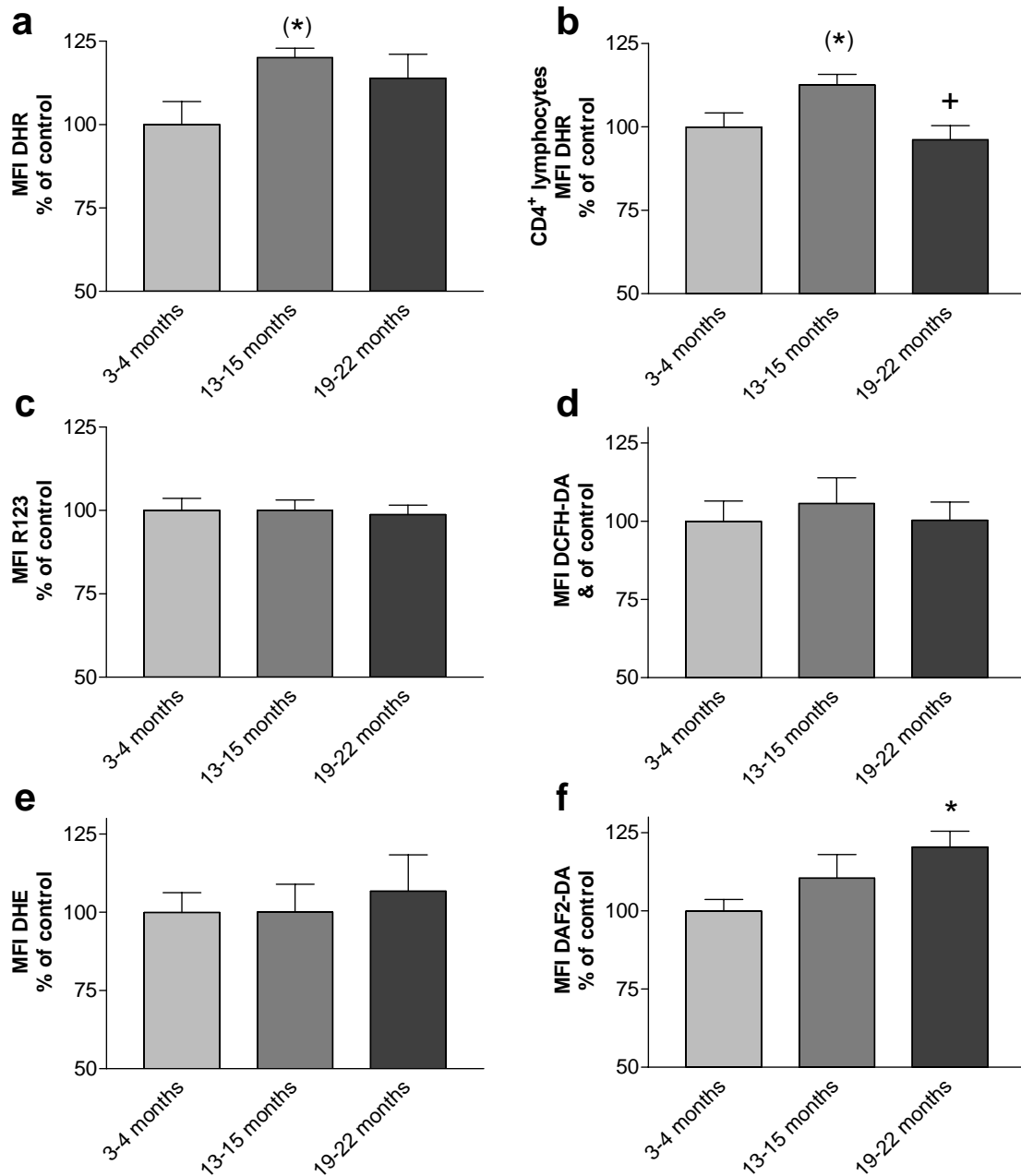


Figure 3.4: Levels of ROS in splenic lymphocytes from C57BL/6J mice during aging.

ROS levels were determined in splenic lymphocytes from young (3 months old, $n = 14$), middle-aged (13-15 months old, $n = 7$) and aged (19-22 months old, $n = 10$) mice. Values are given as percentage of mean fluorescence intensity MFI relative to the mean of 3 months old control mice.

a. Staining with ROS-sensitive dye dihydrorhodamine DHR. One-way ANOVA: n.s. Post hoc t-test (*) $p < 0.1$.

b. Staining of CD4-positive lymphocytes with ROS-sensitive dye dihydrorhodamine DHR. One-way ANOVA: $p = 0.06$. Post hoc t-test (*) $p < 0.1$ vs 3-4 months old mice, + $p < 0.05$ vs 13-15 months old mice.

c. Staining with rhodamine R123. One-way ANOVA: n.s.

d. Staining with ROS-sensitive dye dihydrodichlorofluorescein diacetate DCFH-DA. One-way ANOVA: n.s.

e. Staining with superoxide-sensitive dye dihydroethidium DHE. One-way ANOVA: n.s.

f. Staining with nitric oxide-sensitive dye DAF-2-DA. One-way ANOVA: $p < 0.05$. Post hoc t-test * $p < 0.05$.

3.1.2 Gender differences in ROS metabolism in C57BL/6J mice during aging

During the experiments in this thesis, gender differences in antioxidant parameters in mice during aging were observed. Table 3.2 gives details on the number of animals used for studies of differences between male and female mice in oxidative stress parameters.

Table 3.2: Age groups and numbers of mice used for studies of gender differences during aging.

age (months)	3-4		13-15		19-22	
gender	female	male	female	male	female	male
number of animals	29	12	16	11	11	10

Brains from these mice were analyzed for levels of lipid peroxidation products MDA and HNE and for activities of antioxidant enzymes Cu/Zn-SOD, GPx and GR. Levels of lipid peroxidation product MDA were also studied after *in vitro* stimulation. Splenic lymphocytes were isolated for direct analysis of reactive oxygen species with fluorescent dyes.

3.1.2.1 Gender differences in oxidative damage and enzymatic antioxidant defense in murine brains

In brains from male and female mice, prominent differences in levels of lipid peroxidation products exist. Female mice show lower levels of MDA (Figure 3.5 a) and HNE (Figure 3.5 b) in different age groups. The relative difference was highest for HNE levels in 13-15 months old mice where female mice exhibited only 62 percent of the HNE levels relative to male mice of the same age group, although this marginally failed to reach statistical significance ($p = 0.055$) due to the rather large variation of values.

Analysis of antioxidant enzyme activities revealed that activity of GPx is significantly increased in female compared to male mice (Figure 3.6 b), while no gender differences were observed in activities of Cu/Zn-SOD and GR (Figure 3.6 a and c respectively). Changes in GPx activity ranged from 10 % higher activity in 3-4 months old female mice to a 21 % and 15 % increase in 13-15 months and 19-22 months old mice, respectively.

RESULTS

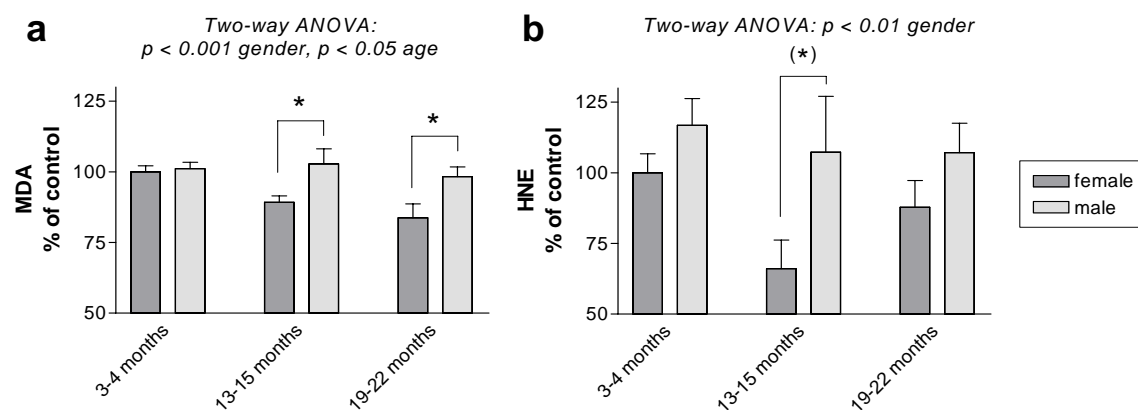


Figure 3.5: Gender differences in levels of lipid peroxidation products in mouse brains during aging.

Lipid peroxidation products were analyzed in each female and male young (3 months, $n = 26$ female, $n = 12$ male), middle-aged (13-15 months, $n = 12$ female, $n = 7$ male) and aged mice (19-22 months old, $n = 11$ female, $n = 9-10$ male). Values are presented as percentage relative to controls (% of 3 months old female mice).

a. Levels of malondialdehyde MDA. Two-way ANOVA $p < 0.001$ effect of gender, $p < 0.05$ effect of age, interaction n.s. Post hoc t-test: * $p < 0.05$ male vs female mice of the same age group.

b. Levels of 4-Hydroxynonenal HNE. Two-way ANOVA $p < 0.01$ effect of gender, effect of age n.s., interaction n.s. Post hoc t-test: (*) $p = 0.055$ male vs female mice of the same age group.

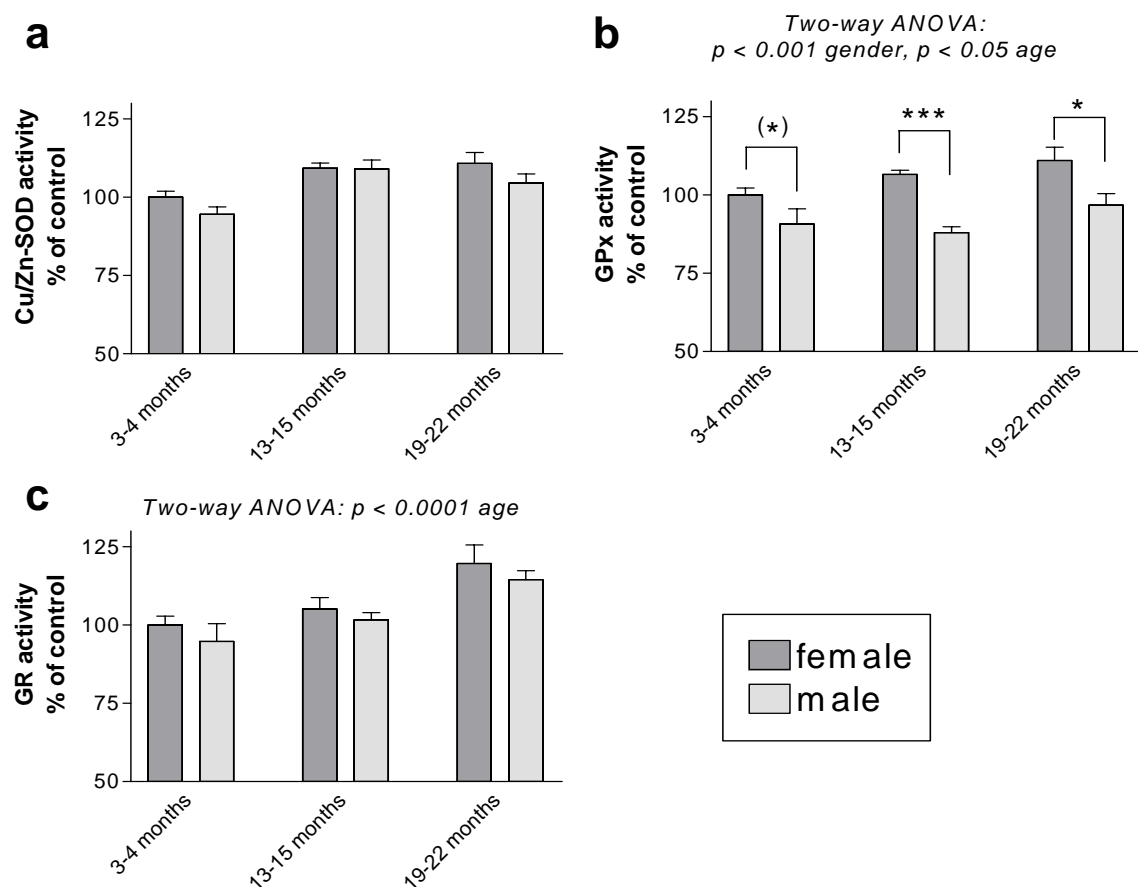


Figure 3.6: Gender differences in activities of antioxidant enzymes in mouse brains during aging.

Legend continued on page 91.

Legend to figure 3.6 (continued):

Enzyme activities were analyzed in each female and male young (3 months, $n = 29$ female, $n = 12$ male), middle-aged (13-15 months, $n = 15-16$ female, $n = 10$ male) and aged mice (19-22 months old, $n = 11$ female, $n = 10$ male). Values are presented as percentage relative to controls (% of 3 months old female mice).

a. Activity of Cu/Zn-superoxide dismutase Cu/Zn-SOD. Two-way ANOVA effect of gender *n.s.*, effect of age $p = 0.06$, interaction *n.s.*

b. Activity of glutathione peroxidase GPx. Two-way ANOVA $p < 0.001$ effect of gender, $p < 0.05$ effect of age, interaction *n.s.* Post hoc *t*-test: (*) $p < 0.1$, * $p < 0.05$, *** $p < 0.001$ male vs female mice of the same age group.

c. Activity of glutathione reductase GR. Two-way ANOVA effect of gender *n.s.*, effect of age $p < 0.0001$, interaction *n.s.*

To test whether this difference in GPx activity might be of functional relevance in protection against ROS, levels of lipid peroxidation product MDA were analyzed after *in vitro* stimulation with FeCl_3 . Interestingly, female mice show lower formation of MDA under these conditions compared to male mice (Figure 3.7).

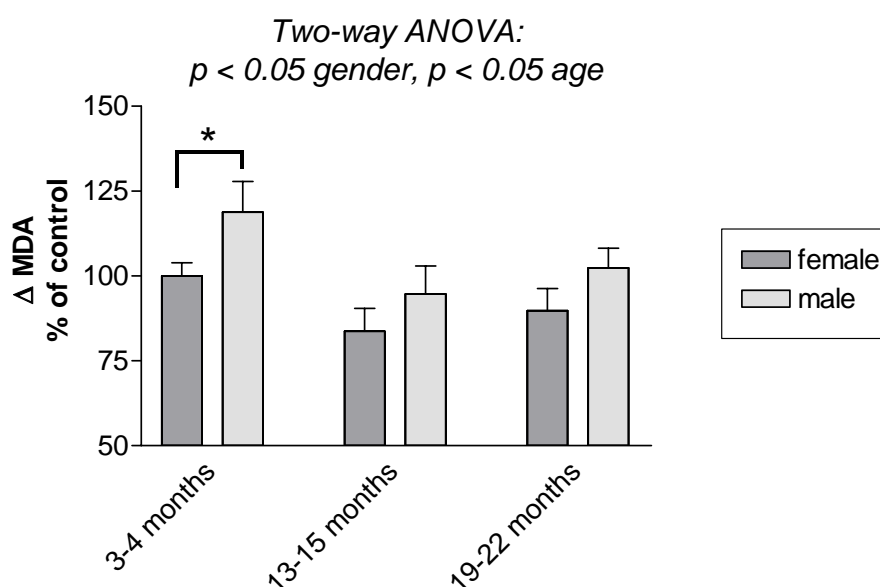


Figure 3.7: Gender differences in formation of lipid peroxidation product MDA after *in vitro* stimulation with FeCl_3 .

Formation of malondialdehyde MDA was stimulated with $50 \mu\text{M}$ FeCl_3 for 30 min at 37°C in brain homogenates from each female and male young (3 month old, $n = 22$ female, $n = 9$ male) mice, middle-aged (13-15 months old, $n = 9$ female, $n = 8$ male) and aged (19-22 months old, $n = 8$ female, $n = 10$ male) mice. Values are presented as percentage relative to controls (% of 3 months old female mice).

Two-way ANOVA: $p < 0.05$ effect of gender, $p < 0.05$ effect of age, interaction *n.s.* Post hoc *t*-test: * $p < 0.05$ male vs female 3 months old mice.

These results indicate that brains from female mice are effectively protected against oxidative damage as evidenced by lower levels of basal as well as stimulated lipid peroxidation products compared to male mice. The higher activity of the antioxidant enzyme GPx in brains from female mice might contribute to these observations.

3.1.2.2 Gender differences in ROS levels in splenic lymphocytes

Apart from gender differences in oxidative parameters in brain tissue, differences between male and female mice were also observed in splenic lymphocytes. In accordance with lower levels of lipid peroxidation products in brains from female mice, female mice show similarly reduced levels of mitochondrial ROS in splenic lymphocytes as evidenced by staining with DHR (Figure 3.8 a). A similar trend was seen for the subgroup of CD4-positive lymphocytes (Figure 3.8 b). This gender difference is not due to different dye retention in cells, which was controlled for by staining with R123 (Figure 3.8 c). Interestingly, levels of cytosolic ROS detected with DCFH-DA and levels of superoxide anions were not changed in female mice compared to male mice (Figure 3.8 d and e respectively). However, we noted a profound gender difference in levels of nitric oxide that was more pronounced in aged mice (Figure 3.8 f).

Since nitric oxide levels are increased in lymphocytes from female mice but DHR oxidation is decreased, it seems unlikely that DHR is oxidized to a significant part by peroxynitrite under these conditions.

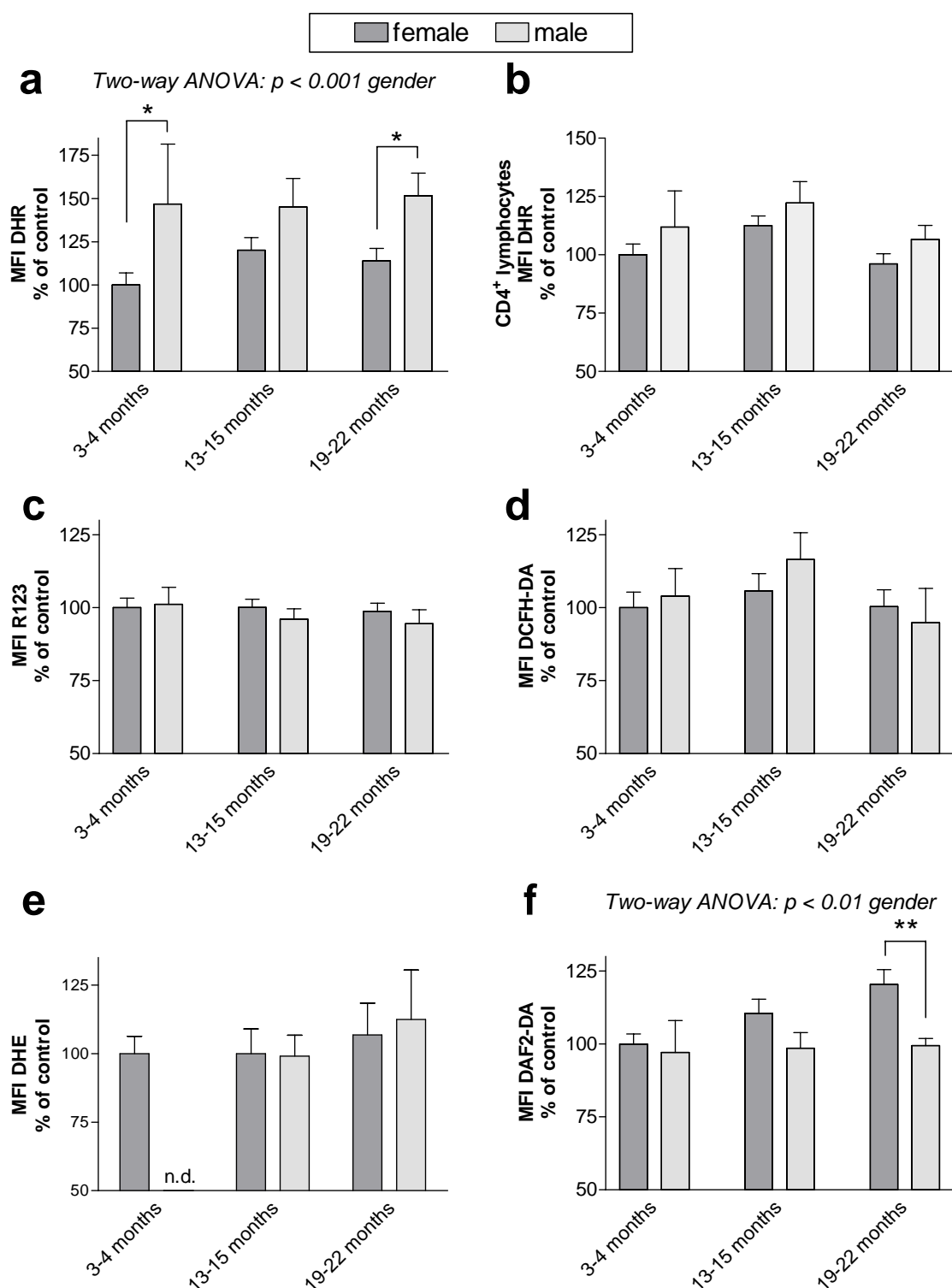
Overall the results indicate that profound gender differences in oxidative parameters exist between female and male mice. Female mice show reduced ROS levels in lymphocytes and also lower levels of lipid peroxidation products in brain tissue suggesting that female mice have lower levels of ROS in several tissues. Moreover, the higher activity of GPx in brains from female mice can significantly contribute to the observed lower levels of lipid peroxidation compared to male mice, supporting the concept that antioxidant enzymes are functionally involved in protection against damage by ROS.

Figure 3.8 (page 93): Gender differences in ROS levels in splenic lymphocytes during aging.

ROS levels were determined in splenic lymphocytes from each female and male young (3-4 months old, $n = 14$ -19 female, $n = 4$ -5 male), middle-aged (13-15 months old, $n = 7$ -13 female, $n = 4$ -9 male) and aged mice (19-22 months old, $n = 8$ female, $n = 10$ male). Values are given as percentage of mean fluorescence intensity MFI relative to the mean of 3-4 months old female control mice.

a. Staining with ROS-sensitive dye dihydrorhodamine DHR. Two-way ANOVA: $p < 0.001$ effect of gender, effect of aging n.s, interaction n.s. Post hoc t-test * $p < 0.05$ female vs male mice of the same age group.

Legend continued on page 93.



Legend to figure 3.8 (continued):

b. Staining of CD4-positive lymphocytes with ROS-sensitive dye dihydrorhodamine DHR. Two-way ANOVA: $p = 0.06$ effect of gender, $p = 0.06$ effect of aging, interaction n.s.

c. Staining with rhodamine R123. Two-way ANOVA: n.s.

d. Staining with ROS-sensitive dye DCFH-DA. Two-way ANOVA: n.s.

e. Staining with superoxide-sensitive dye dihydroethidium DHE. n.d. = not determined. Two-way ANOVA over 13-15 and 19-22 months old age groups n.s.

f. Staining with nitric oxide-sensitive dye DAF-2-DA. Two-way ANOVA: $p < 0.01$ effect of gender, effect of age n.s., interaction n.s. Post hoc t-test ** $p < 0.01$ female vs male 19-22 months old mice.

3.2 ROS metabolism in transgenic AD mouse models

For studies of ROS metabolism in AD transgenic mice, different mouse models (see also 2.1.7.2, page 53 et seqq.) were studied in separate experiments:

- In one large set of experiments, mice transgenic either for (i) human wild-type presenilin 1 (PS1wt), (ii) human mutant presenilin 1 (PS1M146L), (iii) human APP695 with the Swedish, Dutch and London mutations (PDGF-APP) or (iv) double transgenic mice bearing mutant APP and mutant presenilin 1 (PDGF-APP/PS1) were studied. PS1 transgenic mice display increased levels of endogenous rodent A β , but show no formation of A β plaques. Single PDGF-APP transgenic mice form low levels of human A β , however, plaques could not be detected up to an age of 18 months, which was the oldest age group studied (Blanchard et al., 2003). In contrast, double transgenic PDGF-APP/PS1 mice display A β plaques in their brains from an age of 6 months. All mice express the respective transgene in brain tissue which was used for studies of lipid peroxidation products and antioxidant enzyme activities. The PS1wt and PS1M146L animals express the respective transgenic PS1 under the control of the HMG-CoA reductase promotor in brain tissue and in lymphocytes and could be used for direct analysis of ROS in living cells with fluorescent dyes, while expression of the APP transgene under control of the PDGF-promotor was not detectable in lymphocytes excluding APP transgenic mice from these experiments.
- In a different set of experiments, mice bearing mutant APP751 with the Swedish and London mutations under the control of a murine, neuron-specific Thy1-promotor were studied (Thy1-APP). Only brains from these mice were studied since the transgenic APP is not expressed in lymphocytes.

Furthermore, in cooperation with Thomas Bayer and Stephanie Schäfer (Homburg, Saar), brain tissue from APP23 transgenic mice could be assayed for Cu/Zn-SOD activity. Similar to Thy1-APP transgenic mice, APP23 mice express human APP751 with the Swedish double mutation under the control of the murine, neuron-specific Thy1-promotor.

Non-transgenic age- and gender-matched littermate animals (non-tg) were used as controls in all experiments.

3.2.1 ROS metabolism in PDGF-APP and/or PS1 transgenic mice

Brains from non-transgenic littermate mice and PS1wt, PS1M146L, PDGF-APP or PDGF-APP/PS1 transgenic mice at three different age groups were studied for levels of lipid peroxidation products and activities of antioxidant enzymes. Due to the onset of plaque deposition in PDGF-APP/PS1 transgenic mice at 6 months, the age groups were chosen as follows: young (3-4 months old) mice without any A β plaques, middle-aged (13-15 months old) mice of which the double PDGF-APP/PS1 transgenic display moderate plaque load in their brains, and aged (19-22 months old) mice, of which double PDGF-APP/PS1 transgenic mice have widespread plaques. Additionally, levels of ROS were determined in splenic lymphocytes from non-transgenic littermate mice and PS1wt or PS1M146L transgenic mice. Table 3.3 gives details on total numbers and age of animals used for these experiments. Gender differences in lipid peroxidation products and glutathione peroxidase activity in brains and in ROS levels in splenic lymphocytes during aging had been observed previously. Therefore, similar numbers of male and female mice were chosen for each group of transgenic mice in order to avoid possibly confounding gender effects.

Table 3.3: Numbers and age of transgenic animals used for studies.

Transgene	non-tg	PS1wt	PS1M146L	PDGF-APP	PDGF-APP/PS1
age group	number of animals used				
young (3-4 months)	23	11	11	15	12
middle-aged (13-15 months)	27	18	13	15	14
aged (21 months) cohort #1*	7*	-	7*	-	-
aged (19-22 months) cohort #2	21	10	12	7	8

* Only splenic lymphocytes from these animals were studied for ROS levels.

3.2.1.1 Oxidative damage and enzymatic antioxidant defense in transgenic mouse brains

In general, neither levels of lipid peroxidation products MDA and HNE nor activities of antioxidant enzymes were changed in 3-4 months old and 13-15 months old transgenic mice compared to non-transgenic littermate control mice (data not shown).

The only difference between transgenic mice was observed in aged animals, where levels of HNE were increased in PS1M146L compared to PS1wt transgenic mice (Figure 3.9 b).

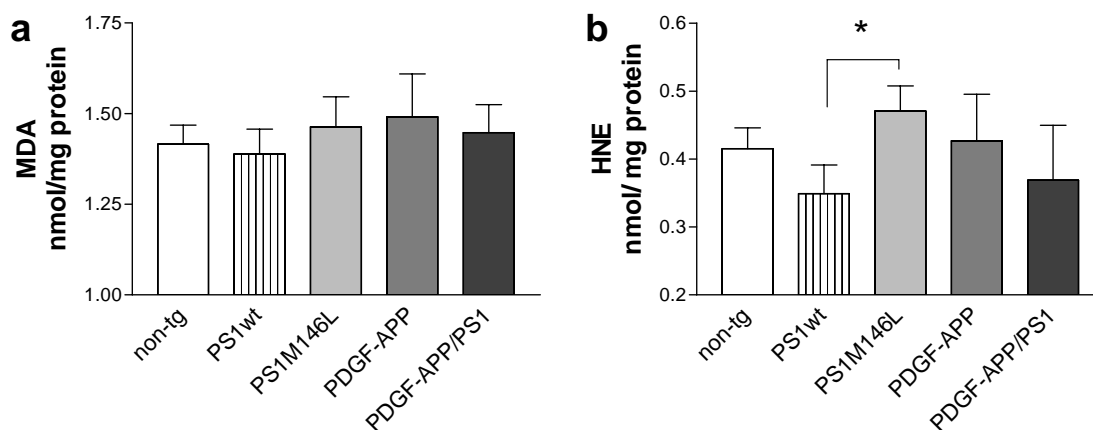


Figure 3.9: Lipid peroxidation products in aged transgenic mice.

Brain homogenates from 19-22 months old non-transgenic littermate control mice (non-tg, $n = 20-21$) and mice transgenic for wildtype PS1 (PS1wt, $n = 9-10$), mutant PS1 (PS1M146L, $n = 11-12$), mutant APP (PDGF-APP, $n = 6-7$) or double transgenic mice expressing both mutant PS1 and mutant APP (PDGF-APP/PS1, $n = 7-8$) were analyzed for MDA and HNE levels.

a. MDA levels (nmol/mg protein). One-way ANOVA *n.s.*

b. HNE levels (nmol/mg protein). One-way ANOVA *n.s.* Post hoc *t*-test * $p < 0.05$ PS1wt vs PS1M146L mice.

Analysis of antioxidant enzyme activities however revealed no differences between transgenic mice (Figure 3.10).

Furthermore, no differences in levels of MDA after *in vitro* stimulation of lipid peroxidation with 50 μ M FeCl₃ could be detected (data not shown).

These results indicate that changes in HNE levels in PS1M146L transgenic mice occur independently of antioxidant enzyme activities.

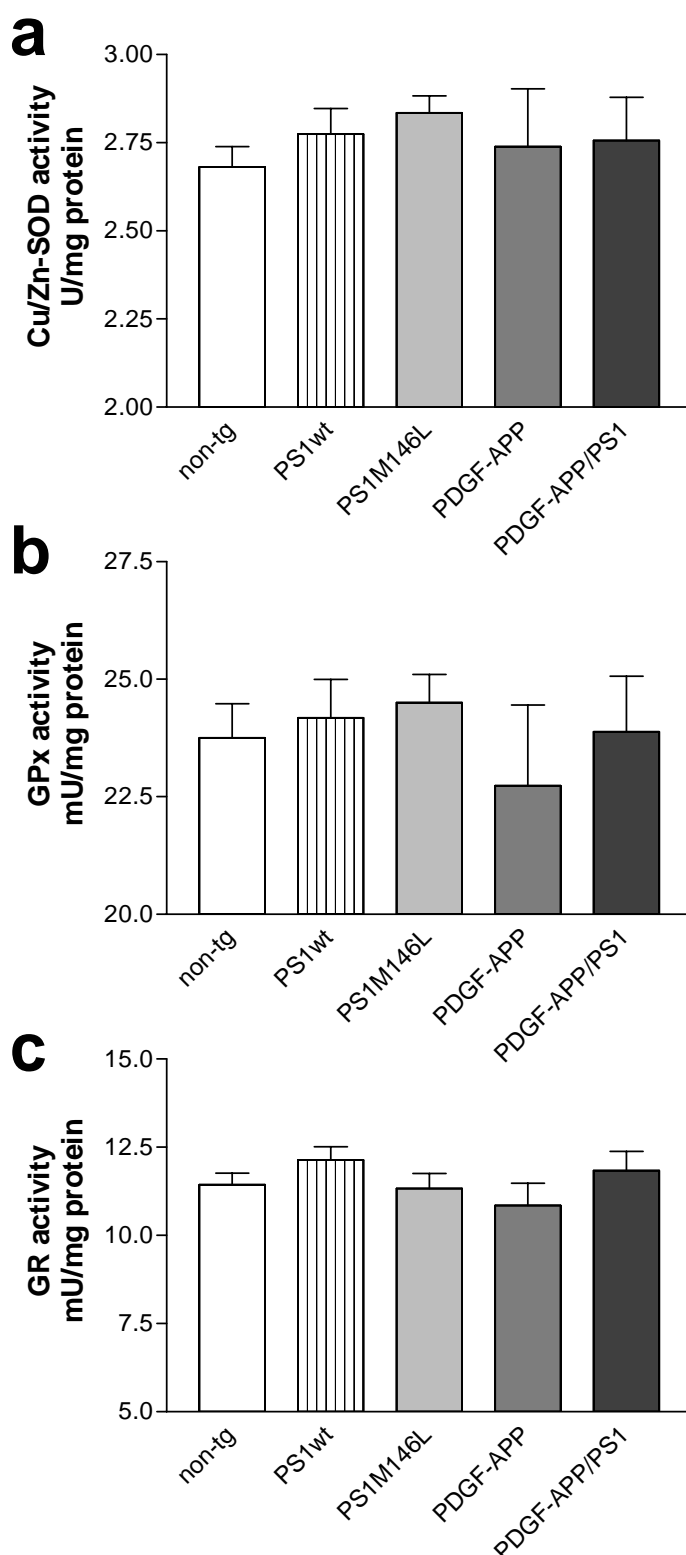


Figure 3.10: Activities of antioxidant enzymes in aged transgenic mice.

Brain homogenates from 19-22 months old non-transgenic littermate control mice (non-tg, $n = 21$) and mice transgenic for wildtype PS1 (PS1wt, $n = 9-10$), mutant PS1 (PS1M146L, $n = 12$), mutant APP (PDGF-APP, $n = 7$) or double transgenic mice expressing both mutant PS1 and mutant APP (PDGF-APP/PS1, $n = 7-8$) were analyzed for activities of antioxidant enzymes Cu/Zn-SOD, GPx and GR.

a. Cu/Zn-SOD activity (U/mg protein). One-way ANOVA *n.s.*

b. GPx activity (mU/mg protein). One-way ANOVA *n.s.*

c. GR activity (mU/mg protein). One-way ANOVA *n.s.*

3.2.1.2 Analysis of splenic lymphocytes from PS1 transgenic mice

3.2.1.2.1 Expression of human PS1wt and PS1M146L in lymphocytes from PS1 transgenic mice

The expression of human PS1 in lymphocytes from transgenic mice has been studied previously at the protein level (Schindowski, 2001). In order to verify PS1 expression in lymphocytes, RNA was isolated from cells and subjected to RT-PCR. DNA products were analyzed in ethidium bromide gels. Figure 3.11 shows a representative agarose gel of PCR products from RNA samples of splenic lymphocytes. As expected, human PS1 is expressed in lymphocytes from PS1wt and PS1M146L transgenic animals, whereas no human transgenic PS1 RNA could be detected in lymphocytes from non-transgenic littermate mice.

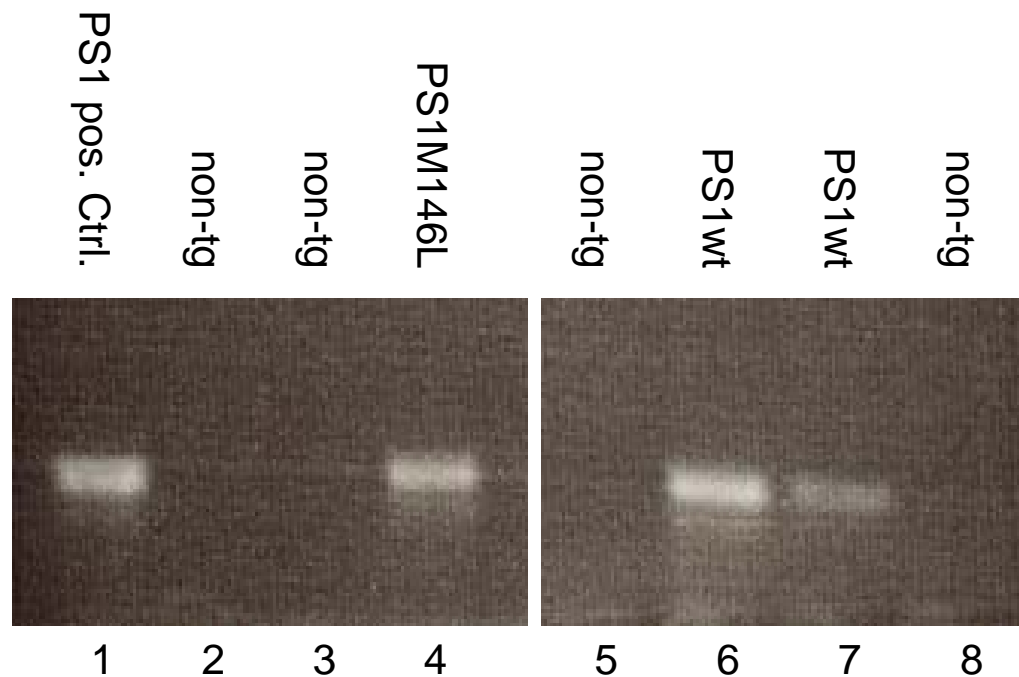


Figure 3.11: Analysis of transgenic PS1 RNA expression in splenic lymphocytes.

RNA samples from splenic lymphocytes derived from PS1M146L or PS1wt transgenic mice (lanes 4, 6, 7) show a PCR product of the same length as the positive control (lane 1). RNA samples from splenic lymphocytes isolated from non-transgenic littermate mice yield no detectable PCR products (lanes 2, 3, 5 and 8).

In contrast to PS1 transgenic mice, no expression of APP could be detected in lymphocytes derived from PDGF-APP or Thy1-APP transgenic mice (data not shown). These APP transgenic mice were therefore excluded from studies of ROS in splenic lymphocytes.

3.2.1.2.2 ROS levels in lymphocytes from PS1 transgenic mice

Splenic lymphocytes from PS1wt and PS1M146L transgenic mice and littermate control mice were isolated and analyzed for ROS levels. Three different age groups were analyzed, of which two cohorts of aged mice were studied (see Table 3.3). Lymphocytes were stained with DHR, which is sensitive mainly for mitochondrial ROS, DCFH-DA, which detects mainly cytosolic ROS, DHE, which is oxidized by superoxide radical anions, or DAF-2-DA, which is selective for nitric oxide. Retention of oxidized DHR in cells was controlled for by staining of lymphocytes directly with R123, the oxidized form of DHR.

In young PS1 transgenic mice at 3-4 months of age, no difference in ROS levels was detected for any of the fluorescent probes used (data not shown).

ROS levels in middle-aged mice

Splenic lymphocytes from middle-aged, 13-15 months old PS1 transgenic mice showed unaltered basal ROS levels (Figure 3.12 a). Similarly, no differences were observed when lymphocytes were stained with other oxidation-sensitive probes DCFH-DA, DHE and DAF-2-DA or R123 under basal conditions in this age group (data not shown). However, DHR oxidation was elevated after serum withdrawal (Figure 3.12 b), which represents a mild stress condition and can trigger oxidative damage in cells (Atabay et al., 1996; Barroso et al., 1997). After further stimulation of lymphocytes with hydrogen peroxide, DHR oxidation was not different between littermate, PS1wt or PS1M146L mice (data not shown).

ROS levels in aged mice, cohort #1

In preliminary experiments, a cohort of 7 non-transgenic littermate control mice and 7 PS1M146L transgenic mice at an age of 21 months were studied for ROS levels detected by staining with oxidation-sensitive DHR. Basal levels of ROS were elevated in lymphocytes from PS1M146L mice (Figure 3.13 a). This increased oxidation of DHR could not be attributed to a single subset of lymphocytes, since increased fluorescence was found in CD3-positive cells (total T-lymphocytes) as well as in subsets of CD4-positive and CD8-positive T-lymphocytes (Figure 3.13 b). Of note, CD4-positive T-lymphocytes display higher DHR oxidation than CD8-positive T-lymphocytes, while intermediate values are detected when CD3-positive lymphocytes, which represent the sum of CD4- and CD8-positive cells, are analyzed.

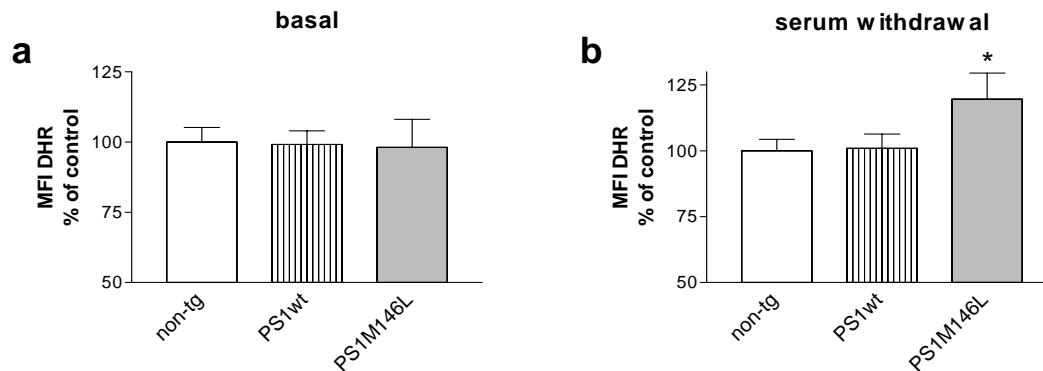


Figure 3.12: Mitochondrial ROS levels in splenic lymphocytes from middle-aged PS1 transgenic mice.

Splenic lymphocytes from 13-15 months old non-transgenic littermate control mice (non-tg, $n = 21-22$) and mice transgenic for wildtype (PS1wt, $n = 11$) or mutant presenilin 1 (PS1M146L, $n = 8-9$) were analyzed for ROS levels with oxidation-sensitive dye DHR.

a. Mean fluorescence intensity MFI of splenic lymphocytes stained with DHR under unstimulated ("basal") conditions expressed as percentage relative to values of non-transgenic littermate control animals. One-way ANOVA: n.s.

b. Mean fluorescence intensity MFI of splenic lymphocytes stained with DHR after serum withdrawal expressed as percentage relative to values of non-transgenic littermate control animals. One-way ANOVA: $p = 0.08$. Post hoc paired t-test * $p < 0.05$ PS1M146L vs non-tg.

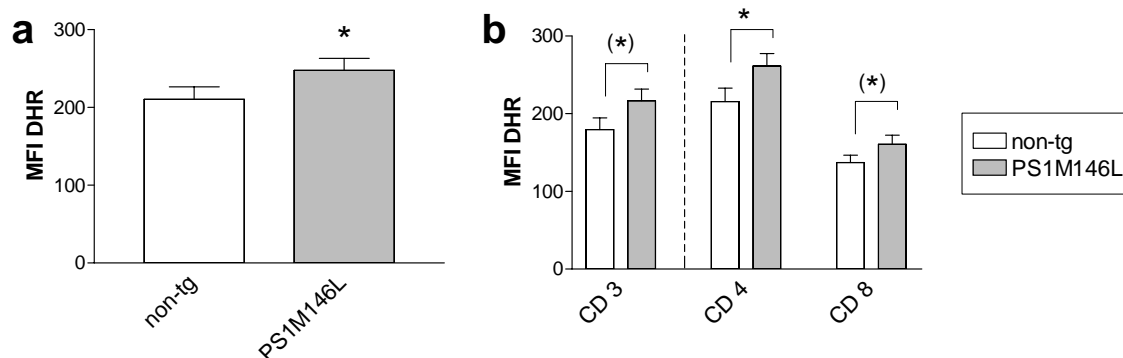


Figure 3.13: Basal levels of mitochondrial ROS in lymphocytes from aged PS1M146L transgenic mice.

Splenic lymphocytes from 21 months old non-transgenic littermate control mice (non-tg, $n = 6-7$) and mice transgenic for mutant presenilin 1 (PS1M146L, $n = 7$) were analyzed for ROS levels with the oxidation-sensitive dye DHR.

a. Mean fluorescence intensity MFI of splenic lymphocytes stained with DHR under unstimulated ("basal") conditions. Paired t-test * $p < 0.05$.

b. Mean fluorescence intensity MFI of splenic lymphocyte subsets stained with DHR under unstimulated conditions. Two-way ANOVA $p < 0.01$ effect of transgene, $p < 0.0001$ difference between subsets, interaction n.s. Post hoc paired t-test (*) $p < 0.1$, * $p < 0.05$ PS1M146L vs non-tg.

Furthermore, DHR oxidation in CD4-positive T-lymphocytes correlated positively and significantly with levels of apoptosis in lymphocytes quantified with 7-AAD staining (apoptosis data from C. Frey; Figure 3.14), indicating that increased ROS formation plays an important role for the apoptotic process in this lymphocyte subset.

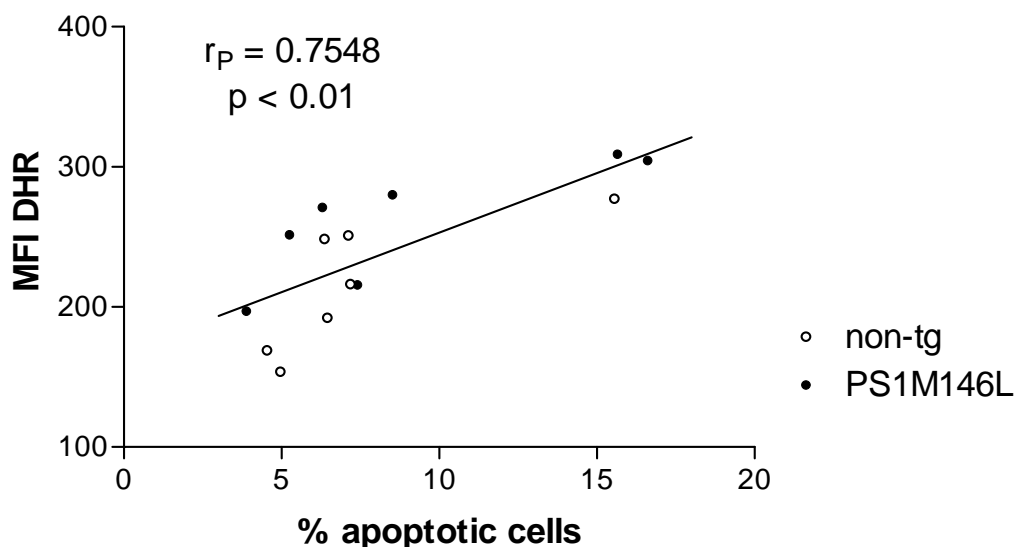


Figure 3.14: Correlation between mitochondrial ROS levels (DHR oxidation) in CD4-positive lymphocytes and apoptosis levels in lymphocytes (7-AAD staining) from aged mice.

CD4-PE antibody-stained splenic lymphocytes from 21 months old non-transgenic littermate control mice (non-tg) and mice transgenic for mutant presenilin 1 (PS1M146L) were analyzed for mitochondrial ROS levels with oxidation-sensitive dye DHR and for percentage of apoptotic cells (7-AAD staining, data from C. Frey). Correlation: $r_p = 0.7548$, $p < 0.01$.

After serum withdrawal, lymphocytes from PS1M146L mice still displayed higher ROS levels (Figure 3.15 a, left bars). Stimulation with 10 or 100 μM hydrogen peroxide induced DHR oxidation in a dose-dependent manner in cells from non-transgenic as well as from PS1M146L mice. The concentrations of H_2O_2 have been chosen according to reports that up to 100 μM have been measured in extracellular fluids under experimental conditions (Hyslop et al., 1995). Although ROS levels were increased in PS1M146L lymphocytes after stimulation with hydrogen peroxide, this effect failed to reach statistical significance (Figure 3.15 a, middle and right bars). Similar effects were observed after stimulation of cells for 4 hours with d-ribose, an agent that induces apoptosis via glutathione depletion (Barbieri et al., 1994; Kletsas et al., 1998). 10 mM and especially 50 mM d-ribose induced a large increase in DHR oxidation after 4 hours of incubation (Figure 3.15 b). Although unstimulated ROS levels after 4 hours of incubation showed a trend towards increased levels in cells from PS1M146L compared to non-transgenic mice, differences were again not significant after stimulation with d-ribose (Figure 3.15 b).

In summary, the relative difference in DHR oxidation between lymphocytes from non-transgenic compared to PS1M146L transgenic animals that was already observed under

RESULTS

basal conditions, was not increased after stimulation with any of the oxidizing agents hydrogen peroxide or d-ribose, suggesting that cells react similarly upon oxidative stimulation, independent of the transgene.

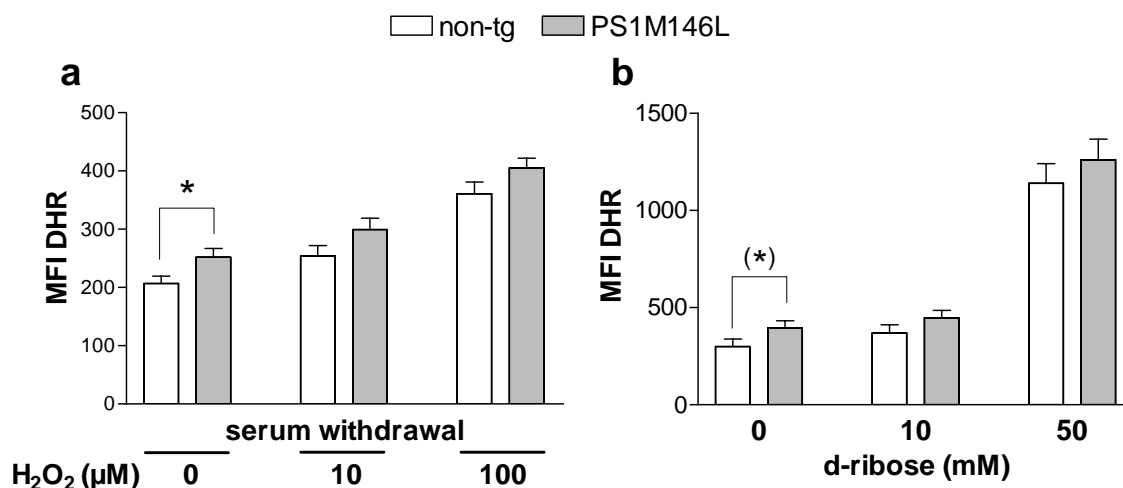


Figure 3.15: ROS levels in lymphocytes from aged PS1M146L transgenic mice after stimulation with hydrogen peroxide or d-ribose.

Splenic lymphocytes from 21 months old non-transgenic littermate control mice (non-tg, $n = 7$) and mice transgenic for mutant presenilin 1 (PS1M146L, $n = 7$) were analyzed for ROS levels with oxidation-sensitive dye DHR.

a. Mean fluorescence intensity MFI of splenic lymphocytes stained with DHR after serum withdrawal and stimulation with hydrogen peroxide for 15 minutes. Two-way ANOVA $p < 0.01$ effect of transgene, $p < 0.0001$ effect of stimulation, interaction n.s. Post hoc paired t-test * $p < 0.05$ PS1M146L vs non-tg.

b. Mean fluorescence intensity MFI of splenic lymphocyte subsets stained with DHR after stimulation with d-ribose for 4 hours. Two-way ANOVA $p < 0.1$ effect of transgene, $p < 0.0001$ effect of stimulation, interaction n.s. Post hoc paired t-test (*) $p < 0.1$ PS1M146L vs non-tg.

ROS levels in aged mice, cohort #2

In subsequent experiments, ROS formation was studied in more detail with various oxidation-sensitive dyes in lymphocytes in a larger cohort of 19-22 months old non-transgenic littermate control animals (non-tg) and mice transgenic for either human wildtype PS1 (PS1wt) or mutant PS1 (PS1M146L).

As already observed in cohort #1, basal DHR oxidation is increased in lymphocytes from PS1M146L mice, but not from PS1wt mice (Figure 3.16 a). Also, retention of R123, which represents the oxidized form of DHR, is slightly increased in cells from PS1M146L mice (Figure 3.16 b), which might to a minor extent contribute to the observed increase in fluorescence after DHR staining. We additionally controlled for this effect by staining lymphocytes with another oxidation-sensitive dye, DCFH-DA, which is not retained in mitochondria and for which a transport by P-glycoprotein has

not been described so far. Similar to DHR oxidation, DCFH-DA is also increasingly oxidized in PS1M146L cells (Figure 3.16 c). Furthermore, fluorescence values obtained after staining with DHR and DCFH-DA were significantly correlated in littermate, PS1wt and PS1M146L animals (Figure 3.16 d), indicating that they detect similar or related ROS in the transgenic animals. Formation of superoxide radical anions is however not increased in lymphocytes from transgenic mice (Figure 3.16 e), and levels of nitric oxide are only slightly increased in PS1M146L mice (Figure 3.16 f), again suggesting that DHR and DCFH-DA are not oxidized by peroxynitrite to a significant extent under our conditions.

Increased oxidation of DHR in lymphocytes from PS1M146L mice was also found after serum withdrawal and stimulation with various concentrations of hydrogen peroxide (Figure 3.17). Of note, even lymphocytes from PS1wt mice showed a tendency towards increased DHR oxidation after stimulation with a relatively low hydrogen peroxide concentration of 1 μ M (Figure 3.17 b). However, DHR oxidation was more pronounced and significantly increased at all concentrations of hydrogen peroxide in lymphocytes from PS1M146L transgenic mice.

When comparing the stimulation data after hydrogen peroxide challenge with basal levels of DHR oxidation, it is obvious that stimulated DHR oxidation is not exacerbated in PS1M146L transgenic mice. This indicates that lymphocytes from PS1M146L mice already display elevated DHR oxidation under unstimulated conditions, but that lymphocytes react similarly upon stimulation independent of the transgene. This is in good accordance with the data obtained on brain tissue, where MDA formation was also not increased in PS1M146L mice after stimulation.

Interestingly, oxidation of DHR in splenic lymphocytes was positively correlated with levels of the lipid peroxidation product HNE in brains from littermate control, PS1wt and PS1M146L mice (Figure 3.18), suggesting that the oxidative stress situation in brain tissue from these animals is also reflected in splenic lymphocytes.

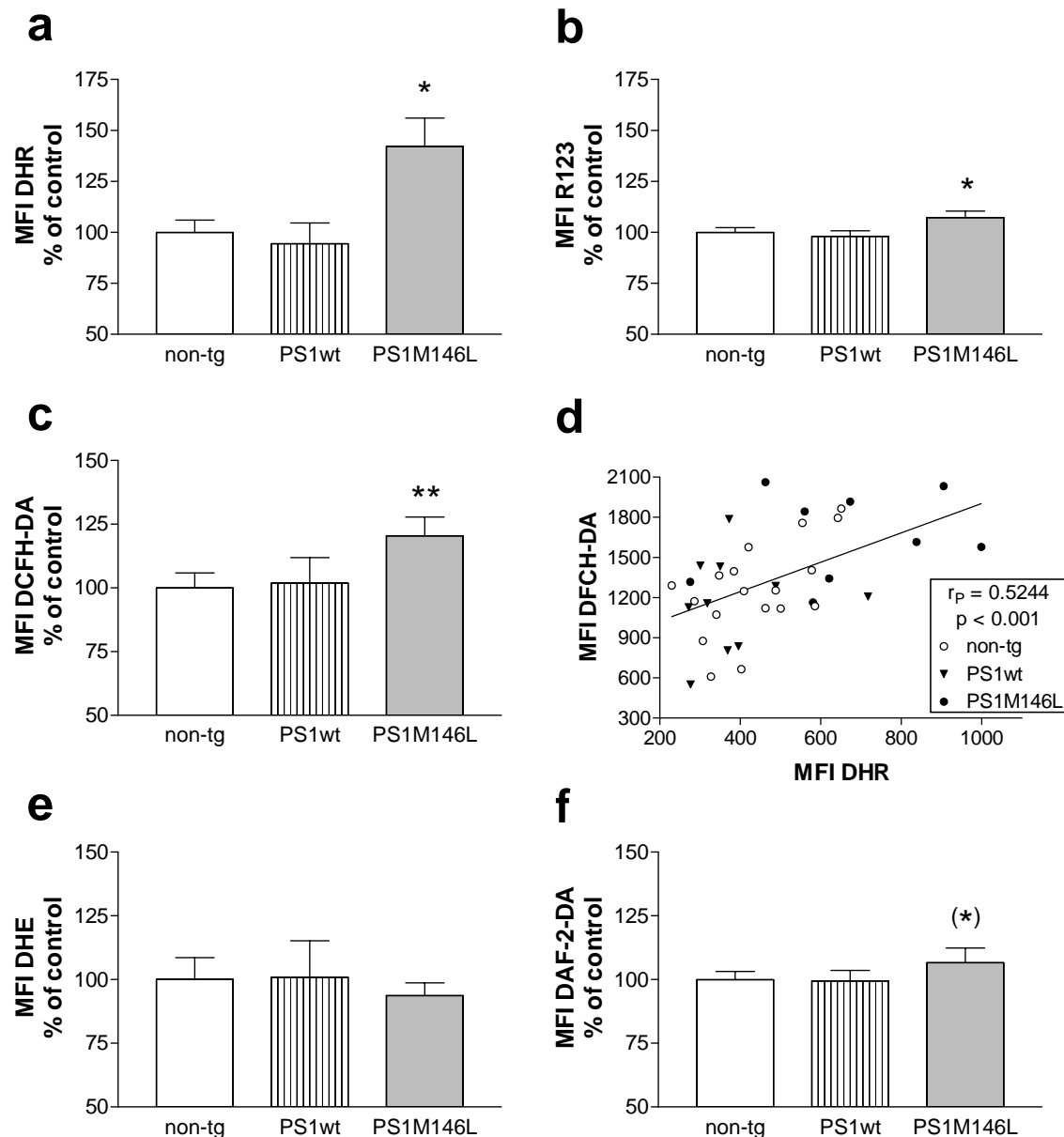


Figure 3.16: Basal levels of various ROS in lymphocytes from PS1wt and PS1M146L transgenic mice.

Splenic lymphocytes from 19-22 months old non-transgenic littermate control mice (non-tg, $n = 20$) and mice transgenic for human wildtype PS1 (PS1wt, $n = 10$) or mutant PS1 (PS1M146L, $n = 11$) were analyzed under unstimulated conditions. Values are given as percentage of mean fluorescence intensity MFI relative to the mean of age-matched non-transgenic littermate control mice (non-tg = 100 %).

a. Staining with ROS-sensitive dye DHR. One-way ANOVA $p < 0.01$. Post hoc paired t-test * $p < 0.05$ PS1M146L vs non-tg.

b. Staining with R123. One-way ANOVA $p < 0.1$. Post hoc paired t-test * $p < 0.05$ PS1M146L vs non-tg.

c. Staining with ROS-sensitive dye DCFH-DA. One-way ANOVA n.s. Post hoc paired t-test ** $p < 0.01$ PS1M146L vs non-tg.

d. Correlation between DHR and DCFH-DA oxidation in lymphocytes from non-transgenic (non-tg) littermate, PS1wt and PS1M146L mice, $p < 0.001$, $r_p = 0.5244$.

e. Staining with superoxide-sensitive dye DHE. One-way ANOVA n.s.

f. Staining with nitric oxide-sensitive dye DAF-2-DA. One-way ANOVA n.s. Post hoc paired t-test (*) $p < 0.1$ PS1M146L vs non-tg.

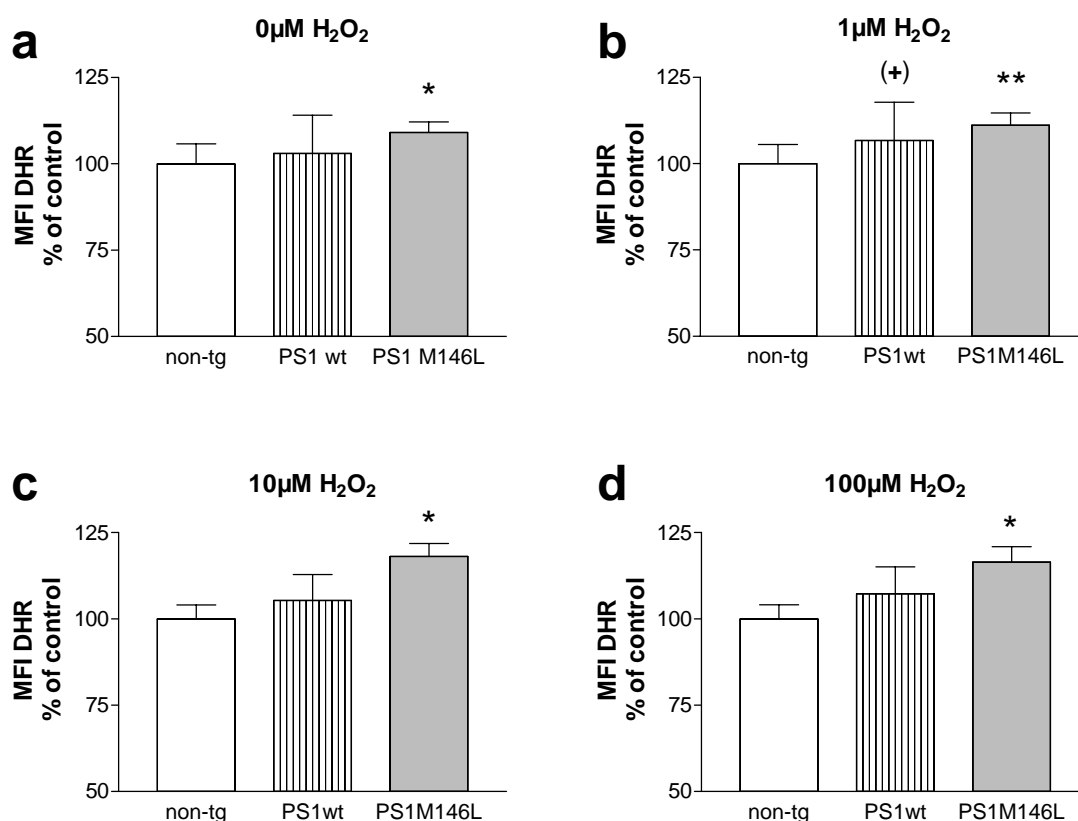


Figure 3.17: ROS levels in lymphocytes from PS1wt and PS1M146L transgenic mice after serum withdrawal and stimulation with hydrogen peroxide.

Splenic lymphocytes from 19-22 months old non-transgenic littermate control mice (non-tg, $n=20$) and mice transgenic for human wildtype (PS1wt, $n=10$) or mutant presenilin 1 (PS1M146L, $n=11$) were analyzed after serum withdrawal and stimulation with hydrogen peroxide H_2O_2 at various concentrations for 15 minutes. Cells were stained with DHR and values are presented as percentage of the mean fluorescence intensity relative to values from non-transgenic littermate controls (non-tg = 100 %).

a. DHR oxidation after serum withdrawal. One-way ANOVA n.s. Post hoc paired t-test * $p < 0.05$ PS1M146L vs non-tg.

b. Stimulation with 1 μM hydrogen peroxide. One-way ANOVA n.s. Post hoc paired t-test (+) $p < 0.1$ PS1wt vs non-tg, ** $p < 0.01$ PS1M146L vs non-tg.

c. Stimulation with 10 μM hydrogen peroxide. One-way ANOVA $p < 0.05$. Post hoc paired t-test * $p < 0.05$ PS1M146L vs non-tg.

d. Stimulation with 100 μM hydrogen peroxide. One-way ANOVA $p < 0.1$. Post hoc paired t-test * $p < 0.05$ PS1M146L vs non-tg.

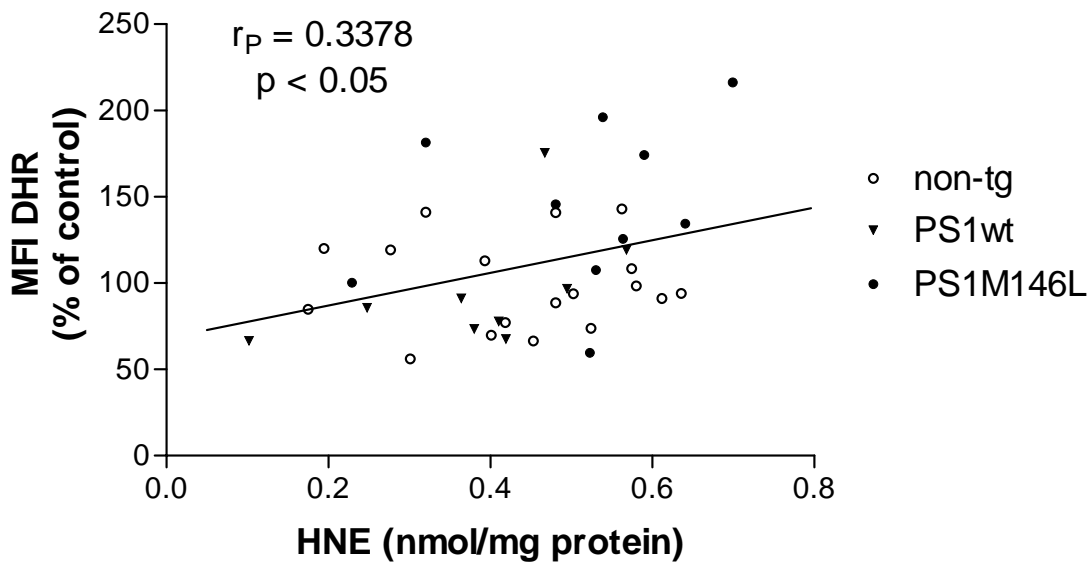


Figure 3.18: Correlation between DHR oxidation in splenic lymphocytes and HNE levels in brains from non-transgenic littermate control mice and mice transgenic for PS1wt and PS1M146L.

Mean fluorescence intensity MFI of lymphocytes stained with DHR (expressed as percentage of values relative to controls) plotted against the concentration of lipid peroxidation product HNE (nmol/mg protein) in brain homogenates from non-transgenic littermate control mice, PS1wt mice and PS1M146L mice. Correlation: $r_P = 0.3378$, $p < 0.05$.

In summary, mutant PS1 leads to elevated ROS levels which were correlated with apoptosis in peripheral cells and increased HNE levels in brain tissue from transgenic mice. The increased HNE levels in brains from these mice can not be explained by altered antioxidant enzyme activities of Cu/Zn-SOD, GPx or GR. Moreover, brain tissue and splenic lymphocytes from PS1M146L transgenic mice did not react more sensitive towards oxidative stimuli, indicating that increased ROS levels and oxidative damage do not arise from impaired antioxidant defense. Of note, no differences in oxidative stress parameters could be detected in mice transgenic for mutant APP (PDGF-APP) or double APP and PS1 mutant transgenic mice (PDGF-APP/PS1).

3.2.2 ROS metabolism in brains from Thy1-APP transgenic mice

Oxidative stress parameters were furthermore studied in another AD transgenic mouse model, Thy1-APP mice, which displays high formation and accumulation of human A β . These mice show A β plaques in their brains from an age of 6 months, therefore mice at an age of 3 months, i.e. before plaque deposition, and mice at ages of 12 and 18 months, i.e. with widespread plaque deposition, were studied. Table 3.4 gives details on the total numbers and gender distribution of animals used in this study.

Table 3.4: Numbers and gender distribution of Thy1-APP and non-transgenic animals used.

Transgene	non-transgenic			Thy1-APP		
age group	total n	female	male	total n	female	male
young (3 months)	18	14	4	15	12	3
middle-aged (12 months)	14	8	6	10	6	4
aged (18 months)	5	-	5	6	-	6

Since the APP transgene is not expressed in lymphocytes from these mice, only brains were studied for markers of lipid peroxidation and antioxidant enzyme activities.

3.2.2.1 ROS metabolism in brains from Thy1-APP transgenic mice during aging

As already observed in aging studies of murine brains (see 3.1.1.1), levels of lipid peroxidation products MDA and HNE decline with rising age (Figure 3.19). The APP transgene did not affect the levels of lipid peroxidation product MDA (Figure 3.19 a), levels of HNE were however highly and significantly increased in 12 months old Thy1-APP transgenic animals compared to age-matched controls (Figure 3.19 b).

In order to assess a possible contribution of antioxidant defense to the observed changes in HNE levels, activities of antioxidant enzymes were assayed. The activity of Cu/Zn-SOD is impaired in 3 and 12 months old Thy1-APP transgenic mice (Figure 3.20 a), which was significant only for the 12 month age group. Activities of GPx and GR were however not affected by the APP transgene (Figure 3.20 b and c, respectively). Activity of Cu/Zn-SOD showed a trend towards higher activity in aged animals (Two-way ANOVA $p = 0.08$ effect of age), and GR activity was significantly increased during aging (Two-way ANOVA $p < 0.0001$ effect of age). Similar results have been obtained before in aging studies (see chapter 3.1.1.1, page 82 et seqq.). The lack of significant upregulation of GPx activity during aging in this cohort is caused by the fact that the 18 months old group consisted only of male animals. As male mice consistently display lower activity of GPx, the aging effect could not be detected in this cohort.

Despite the reduced Cu/Zn-SOD activity, levels of stimulated MDA were not significantly different between non-transgenic and Thy1-APP transgenic mice in any of the age groups (Figure 3.21).

In summary, these results suggest that the reduced Cu/Zn-SOD activity in 12 months old Thy1-APP transgenic mice could be causally related to the increased basal HNE levels. However, impaired Cu/Zn-SOD activity does probably not lead to alterations in MDA formation during stimulation of lipid peroxidation *in vitro*.

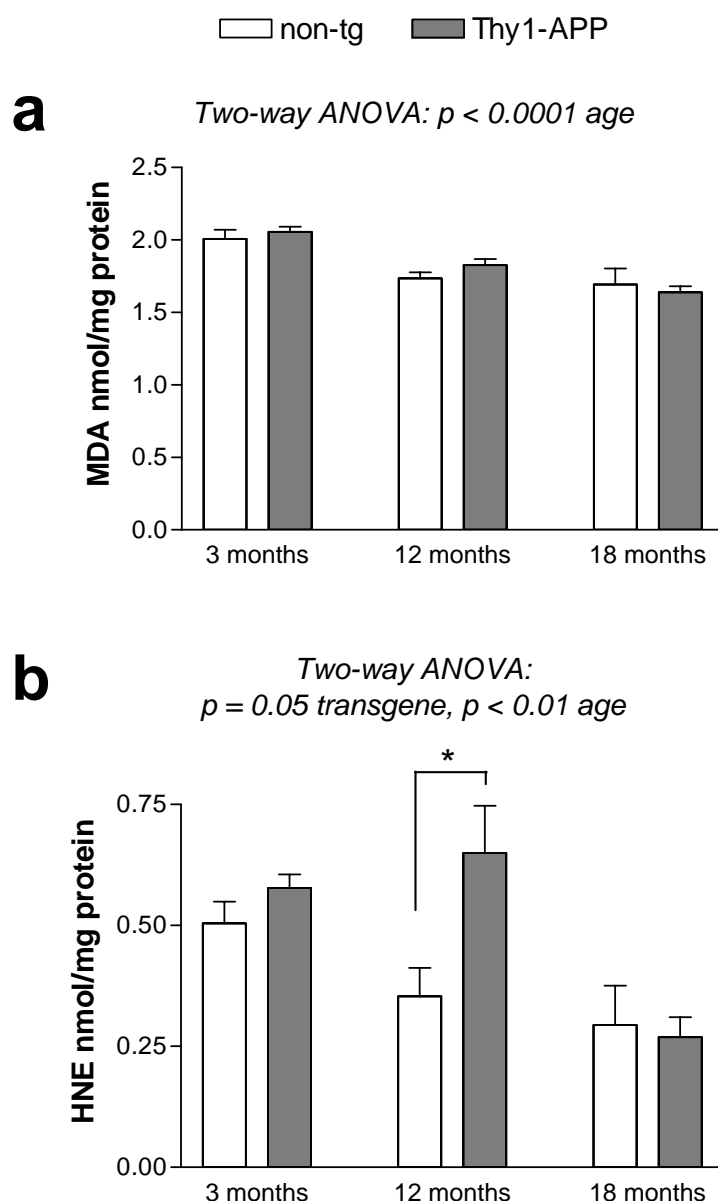


Figure 3.19: Levels of lipid peroxidation products in Thy1-APP transgenic mice during aging.

Lipid peroxidation products MDA and HNE were determined in brain homogenates from 3, 12 and 18 months old non-transgenic littermate control animals (non-tg: white bars, 3 months $n = 9-10$, 12 months $n = 14$, 18 months $n = 5$) and age-matched Thy1-APP transgenic mice (Thy1-APP: grey bars, 3 months $n = 7$, 12 months $n = 9$, 18 months $n = 6$).

a. Levels of MDA (nmol/mg protein). Two-way ANOVA: effect of transgene *n.s.*, $p < 0.0001$ effect of age, interaction *n.s.*

b. Levels of HNE (nmol/mg protein). Two-way ANOVA: effect of transgene $p = 0.05$, effect of age $p < 0.01$, interaction $p = 0.08$. Post hoc *t*-test: * $p < 0.05$.

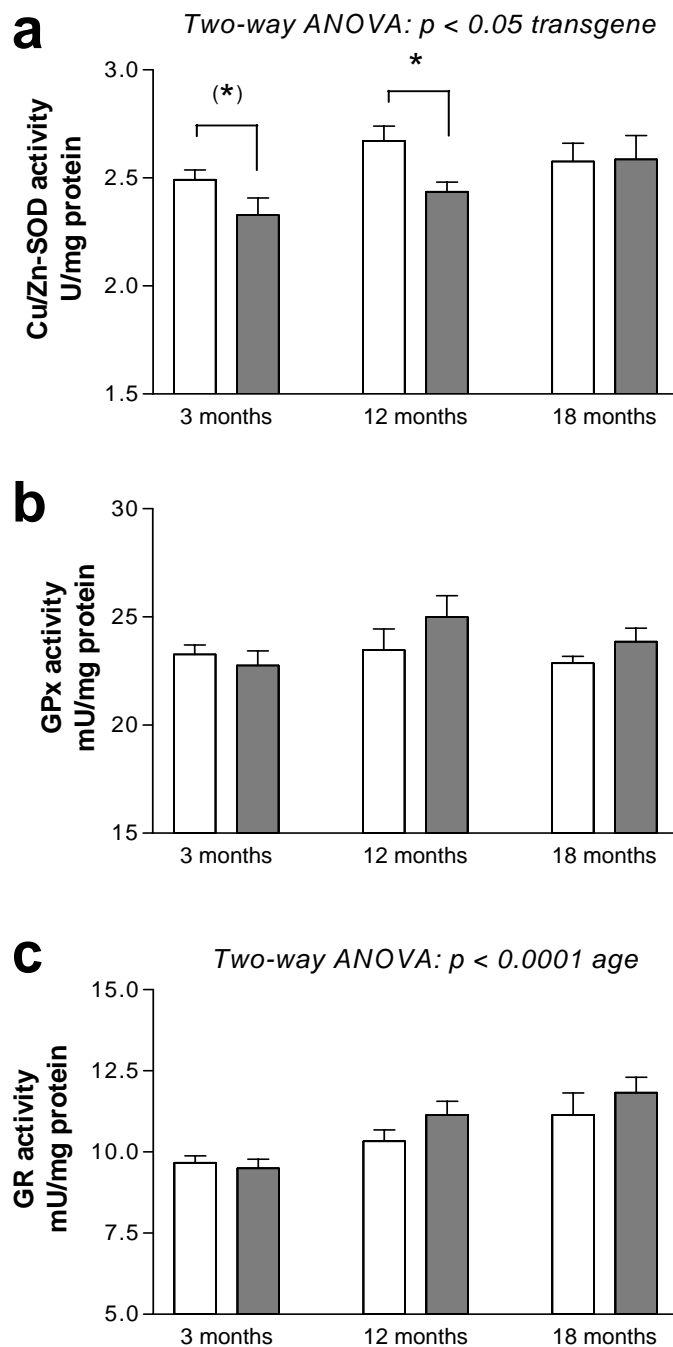


Figure 3.20: Activities of antioxidant enzymes in Thy1-APP transgenic mice during aging.

Activities of Cu/Zn-SOD, GPx and GR were determined in brain homogenates from 3, 12 and 18 months old non-transgenic littermate control animals (non-tg: white bars, 3 months $n = 17-18$, 12 months $n = 13-14$, 18 months $n = 5$) and age-matched Thy1-APP transgenic mice (Thy1-APP: grey bars, 3 months $n = 15$, 12 months $n = 8-10$, 18 months $n = 6$).

a. Cu/Zn-SOD activity (U/mg protein). Two-way ANOVA: $p < 0.05$ effect of transgene, $p = 0.08$ effect of age, interaction n.s. Post hoc t -test: (*) $p = 0.07$ 3 months old Thy1-APP vs non-tg, * $p < 0.05$ 12 months old Thy1-APP vs non-tg.

b. GPx activity (mU/mg protein). Two-way ANOVA: effect of transgene n.s., effect of age n.s., interaction n.s.

c. GR activity (mU/mg protein). Two-way ANOVA: effect of transgene n.s., effect of age $p < 0.0001$, interaction n.s.

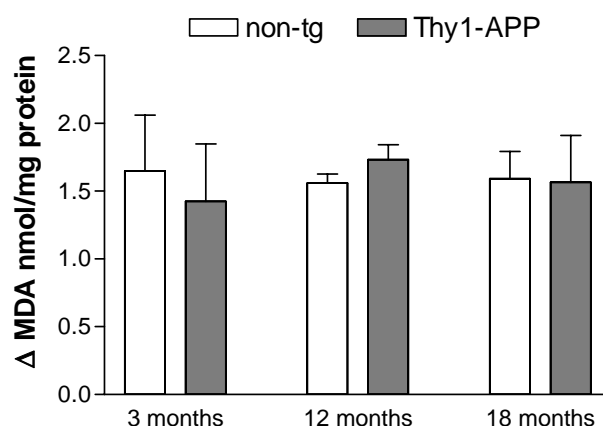


Figure 3.21: Stimulated levels of MDA in Thy1-APP transgenic mice during aging.

Formation of malondialdehyde MDA was stimulated with 50 μ M FeCl₃ for 30 min at 37°C in brain homogenates from non-transgenic littermate control animals (non-tg: white bars, 3 months $n = 10$, 12 months $n = 14$, 18 months $n = 5$) and age-matched Thy1-APP transgenic mice (Thy1-APP: grey bars, 3 months $n = 8$, 12 months $n = 10$, 18 months $n = 6$). Two-way ANOVA: *n.s.*

3.2.2.2 Gender differences in ROS metabolism in Thy1-APP transgenic mice

Analyzing the changes in oxidative stress parameters in Thy1-APP transgenic mice in more detail, the 3 months and 12 months old groups were divided into each male and female subgroups. At 18 months of age, only male animals were studied, which could not be included in this analysis.

At the level of lipid peroxidation parameters, a gender difference was observed in young mice. At 3 months of age, increased levels of MDA and HNE were found only in female Thy1-APP transgenic mice, whereas levels of MDA and HNE were similar in male Thy1-APP transgenic mice compared to controls (Figure 3.22 a and b, left bars). At an age of 12 months, both gender were similarly affected by the APP transgene leading to increased HNE levels in male and female Thy1-APP transgenic mice (Figure 3.22 b, right bars). The relative increase in HNE levels was however higher in 12 months old animals: female and male 12 months old Thy1-APP transgenic mice displayed 86 % or 79 % higher HNE levels, respectively, compared to 36 % higher HNE levels in 3 months old female Thy1-APP mice relative to age- and gender-matched non-transgenic mice. Furthermore – as already observed previously in studies of non-transgenic C57BL/6J mice (3.1.2.1) – female mice displayed lower levels of MDA as well as HNE, irrespective of the transgene.

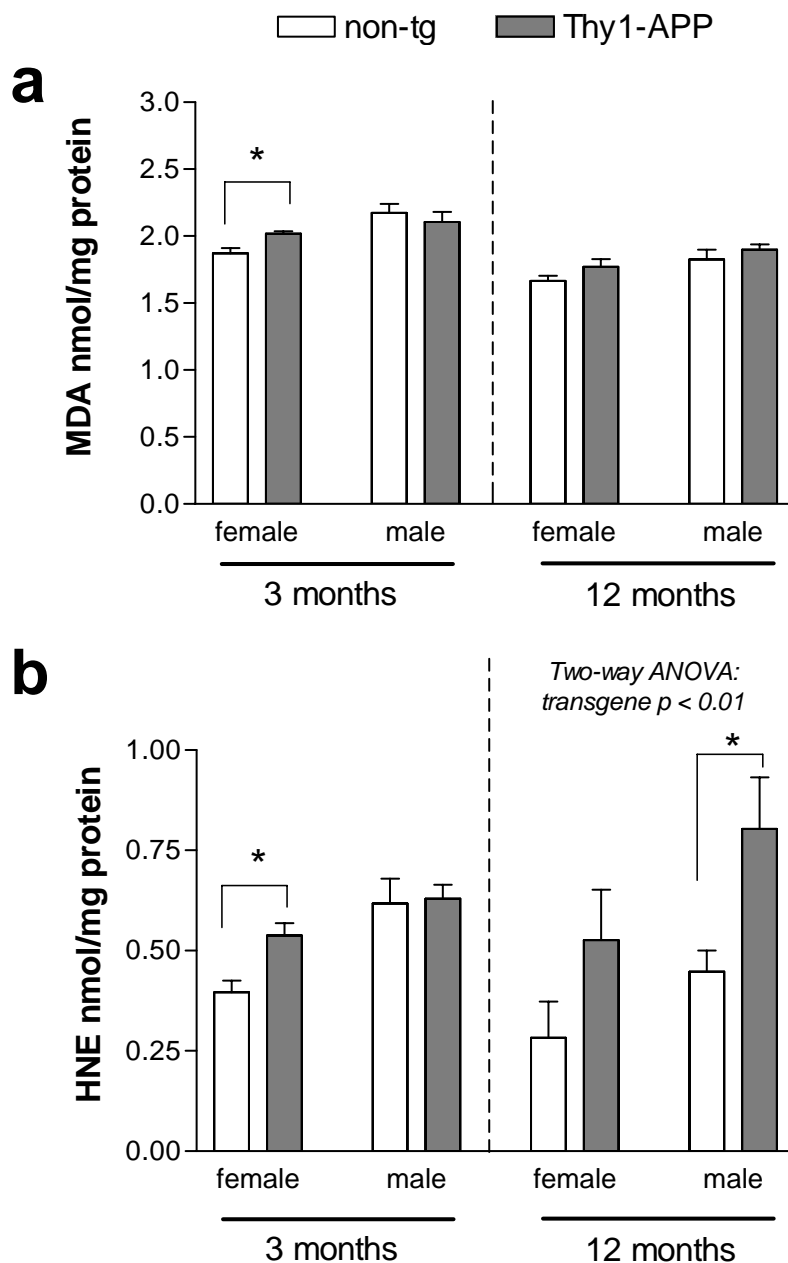


Figure 3.22: Effect of gender on lipid peroxidation parameters in 3 and 12 months old Thy1-APP transgenic mice.

Lipid peroxidation products MDA and HNE were determined in brain homogenates from 3 and 12 months old female and male non-transgenic (non-tg, white bars) littermate control animals and Thy1-APP transgenic mice (Thy1-APP, grey bars). 3 months old female mice $n = 5$ non-tg, $n = 4$ Thy1-APP; 3 months old male mice $n = 4$ non-tg, $n = 3$ Thy1-APP; 12 months old female mice $n = 8$ non-tg, $n = 5$ Thy1-APP; 12 months old male mice $n = 6$ non-tg, $n = 4$ Thy1-APP.

a. Levels of MDA (nmol/mg protein).

3 months old animals: Two-way ANOVA: effect of transgene *n.s.*, $p < 0.01$ effect of gender, interaction $p = 0.05$. Post hoc *t*-test * $p < 0.05$.

12 months old animals: Two-way ANOVA: effect of transgene *n.s.*, effect of gender $p < 0.05$, interaction *n.s.*

b. Levels of HNE (nmol/mg protein).

3 months old animals: Two-way ANOVA: effect of transgene $p = 0.09$, effect of gender $p < 0.01$, interaction *n.s.* Post hoc *t*-test * $p < 0.05$.

12 months old animals: Two-way ANOVA: effect of transgene $p < 0.01$, effect of gender $p < 0.05$, interaction *n.s.* Post hoc *t*-test * $p < 0.05$.

Similarly, the activity of Cu/Zn-SOD in the 3 months old age group was reduced only in female Thy1-APP transgenic mice compared to female non-transgenic mice, whereas male Thy1-APP transgenic mice were not affected at that age (Figure 3.23, left bars). At 12 months of age, both female and male Thy1-APP transgenic mice showed reduced activity of Cu/Zn-SOD compared to non-transgenic mice (Figure 3.23, right bars).

Although the APP transgene did not affect the activities of GPx (Figure 3.24) or GR (data not shown), female mice in general exhibited higher activities of GPx compared to age-matched male mice (Figure 3.24), which was independent of the transgene. This effect had already been observed previously (see 3.1.2.1, page 90).

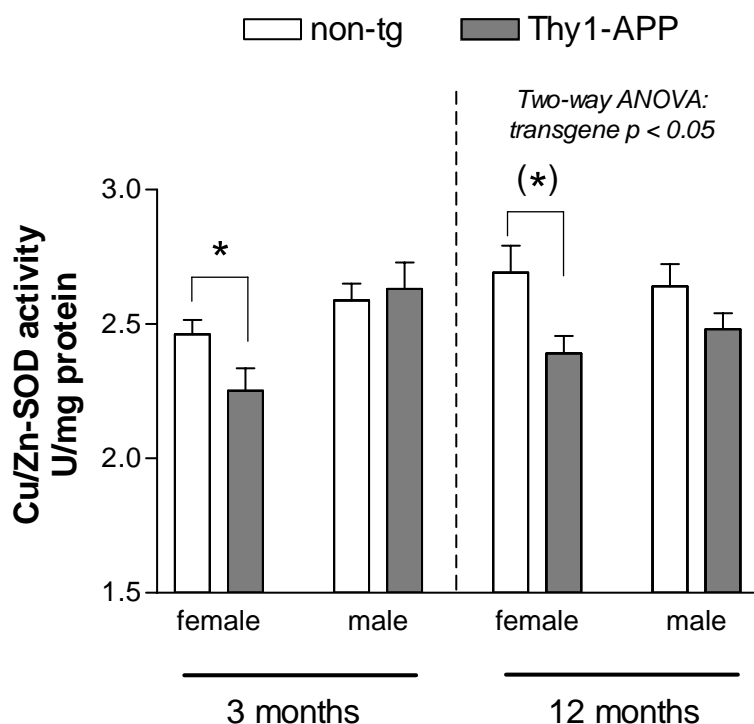


Figure 3.23: Effect of gender on Cu/Zn-SOD activity in 3 and 12 months old Thy1-APP transgenic mice.

Activity of Cu/Zn-SOD was determined in brain homogenates from 3 and 12 months old female and male non-transgenic (non-tg, white bars) littermate control animals and Thy1-APP transgenic mice (Thy1-APP, grey bars). 3 months old female mice $n = 13$ non-tg, $n = 12$ Thy1-APP; 3 months old male mice $n = 4$ non-tg, $n = 3$ Thy1-APP; 12 months old female mice $n = 8$ non-tg, $n = 4$ Thy1-APP; 12 months old male mice $n = 5$ non-tg, $n = 4$ Thy1-APP.

3 months old animals: Two-way ANOVA: effect of transgene *n.s.*, effect of gender $p < 0.05$, interaction *n.s.* Post hoc *t*-test * $p < 0.05$.

12 months old animals: Two-way ANOVA: effect of transgene $p < 0.05$, effect of gender *n.s.*, interaction *n.s.* Post hoc *t*-test (*) $p < 0.1$.

3 months old animals: Two-way ANOVA: n.s.

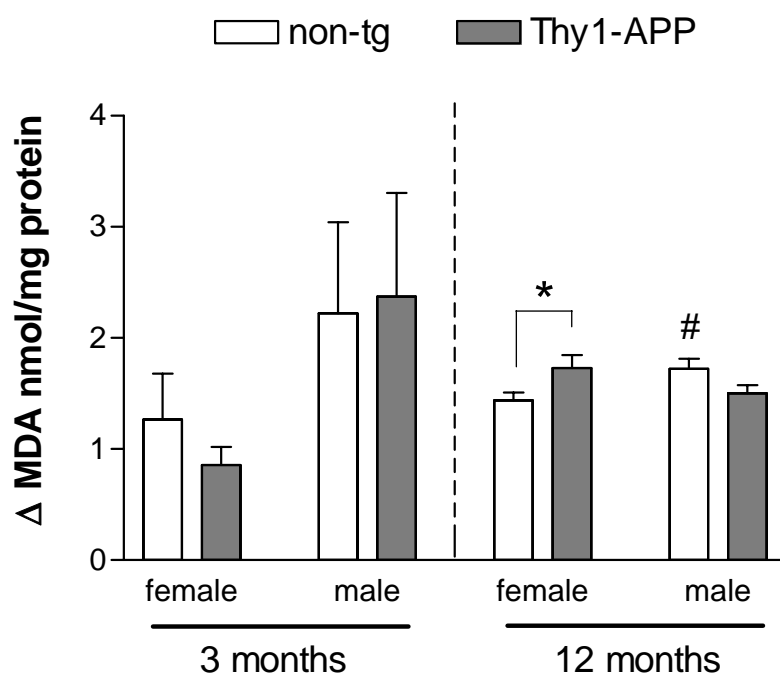


Figure 3.25: Gender differences in stimulated MDA levels in 3 and 12 months old Thy1-APP transgenic mice.

Formation of malondialdehyde MDA was stimulated with 50 μ M FeCl_3 for 30 min at 37°C in brain homogenates from 3 and 12 months old female and male non-transgenic (non-tg, white bars) littermate control animals and Thy1-APP transgenic mice (Thy1-APP, grey bars). 3 months old female mice $n = 6$ non-tg, $n = 5$ Thy1-APP; 3 months old male mice $n = 4$ non-tg, $n = 3$ Thy1-APP; 12 months old female mice $n = 8$ non-tg, $n = 6$ Thy1-APP; 12 months old male mice $n = 6$ non-tg, $n = 3$ Thy1-APP.

3 months old animals: Two-way ANOVA: effect of transgene *n.s.*, $p < 0.05$ effect of gender, interaction *n.s.*

12 months old animals: Two-way ANOVA: effect of transgene *n.s.*, effect of gender *n.s.*, interaction $p < 0.05$. Post hoc *t*-test * $p < 0.05$ female non-tg vs Thy1-APP mice, # $p < 0.05$ female vs male non-tg mice.

In summary, female Thy1-APP transgenic mice were affected by increased HNE levels and reduced Cu/Zn-SOD activity earlier than male transgenic mice. Moreover, at an age of 12 months, only female Thy1-APP transgenic mice are vulnerable towards increased stimulation of MDA formation. These findings suggest a gender-specific earlier and/or higher vulnerability towards oxidative damage in female Thy1-APP transgenic mice compared to male Thy1-APP transgenic mice.

3.2.3 Cu/Zn-SOD activity in brains from APP23 mice

In cooperation with Thomas A. Bayer and Stephanie Schäfer, Homburg/Saar, Germany, brain tissue samples from another APP transgenic mouse model, APP23 mice, could be analysed for Cu/Zn-SOD activity. Similar to the Thy1-APP transgenic mice described above, this mouse model expresses the 751 amino acid form of human APP under the control of the murine Thy1 promotor. The human APP gene contains only the Swedish double mutation (KM670/671NL). These mice develop A β plaques from an age of 6 months (Sturchler-Pierrat et al., 1997). Two groups of mice at an age of 15 and 21 months could be studied. Similar to Thy1-APP transgenic mice, activity of Cu/Zn-SOD was reduced in both age groups (Figure 3.26).

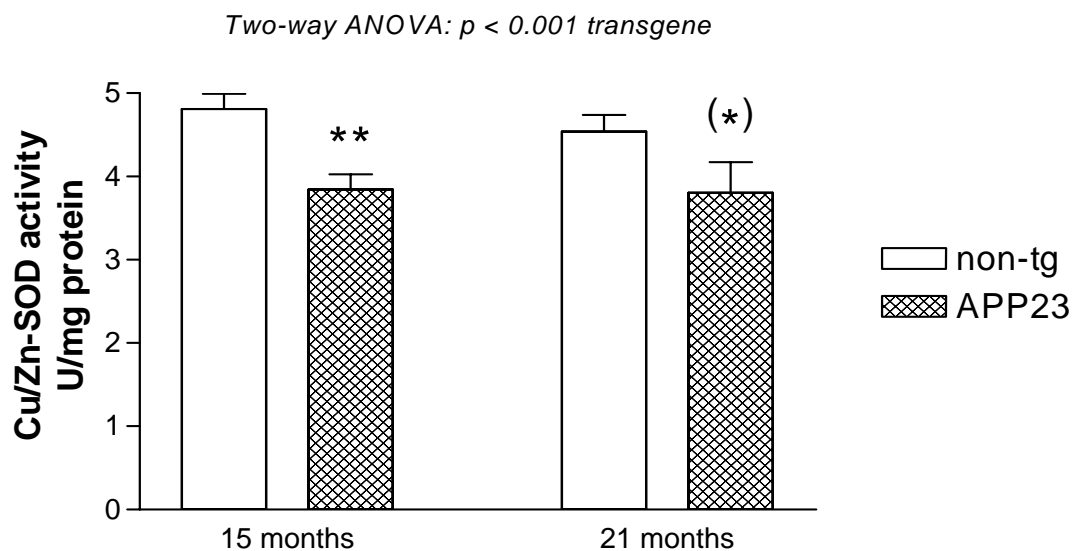


Figure 3.26: Cu/Zn-SOD activity is reduced in brains from APP23 mice.

Activity of Cu/Zn-SOD was determined in brain homogenates from 15 and 21 months old non-transgenic (non-tg, white bars) control animals and APP23 transgenic mice (APP23, streaked bars). 15 months old mice: $n = 14$ non-tg (5 female, 9 male), $n = 11$ APP23 (6 female, 5 male). 21 months old mice: $n = 17$ non-tg (7 female, 10 male), $n = 6$ APP23 (3 female, 3 male). Two-way ANOVA: $p < 0.001$ effect of transgene, effect of age n.s., interaction n.s. Post hoc t -test ** $p < 0.05$ 15 months old APP23 vs non-transgenic mice, (*) $p = 0.08$ 21 months old APP23 vs non-transgenic mice.

In summary, the independent study of APP23 transgenic mouse brains confirms the previous observations in Thy1-APP transgenic mice that overexpression of APP and formation of A β can lead to a reduction in the activity of the antioxidant enzyme Cu/Zn-SOD. This represents a newly identified mechanism by which oxidative toxicity due to APP mutations can be explained.

3.2.4 Comparative analysis of human APP expression and A β expression levels in PDGF-APP, PDGF-APP/PS1 and Thy1-APP mouse models

APP expression and A β levels were analyzed in brain homogenates from PDGF-APP, PDGF-APP/PS1 and Thy1-APP transgenic mice in order to compare A β accumulation between the different mouse models. For these studies, brains were initially homogenized in Tris-buffered saline for extraction of soluble A β followed by subsequent dissolution of the remaining pellet in SDS solution, which solubilizes the remaining insoluble A β fraction. Both Tris- and SDS-lysates were analyzed by Western blot, and Tris-homogenates from PDGF-APP, PDGF-APP/PS1 and Thy1-APP transgenic mouse brains were additionally employed for ELISA quantification of soluble A β_{1-40} levels.

The numbers and gender distribution of the respective transgenic animals used in this study are given in Table 3.5.

Table 3.5: Numbers and gender distribution of different APP transgenic mice used for analysis of APP expression and A β formation.

Transgene	PDGF-APP		PDGF-APP/PS1		Thy1-APP	
	female	male	female	male	female	male
3-4 months	1	1	1	1	4	3
7 months	-	-	-	-	2	-
12 months	-	-	-	-	6	4
13-15 months	1	1	3	2	-	2
18-22 months	1	1	3	3	-	2

3.2.4.1 Western blot analysis of human APP and A β expression

The antibody W0-2 is directed against the human A β sequence and hence detects full-length human APP at approximately 100 kDa, soluble sAPP α at approximately 90 kDa, the C99 peptide at 12 kDa and the A β peptide at 4 kDa simultaneously. This allows for the convenient analysis of AD-relevant processes of APP cleavage at the same time in a single blot. However, sAPP α staining was very weak and therefore not analysed.

RESULTS

Thy1-APP transgenic mice showed an age-associated elevation in the levels of soluble A β in Tris-soluble brain extracts, while expression of full-length APP during aging remained unchanged (Figure 3.27). Of note, some inter-individual variability in soluble A β exists in mice of the same age, despite quite homogenous expression of APP. There was also a slight increase in C99 levels with aging.

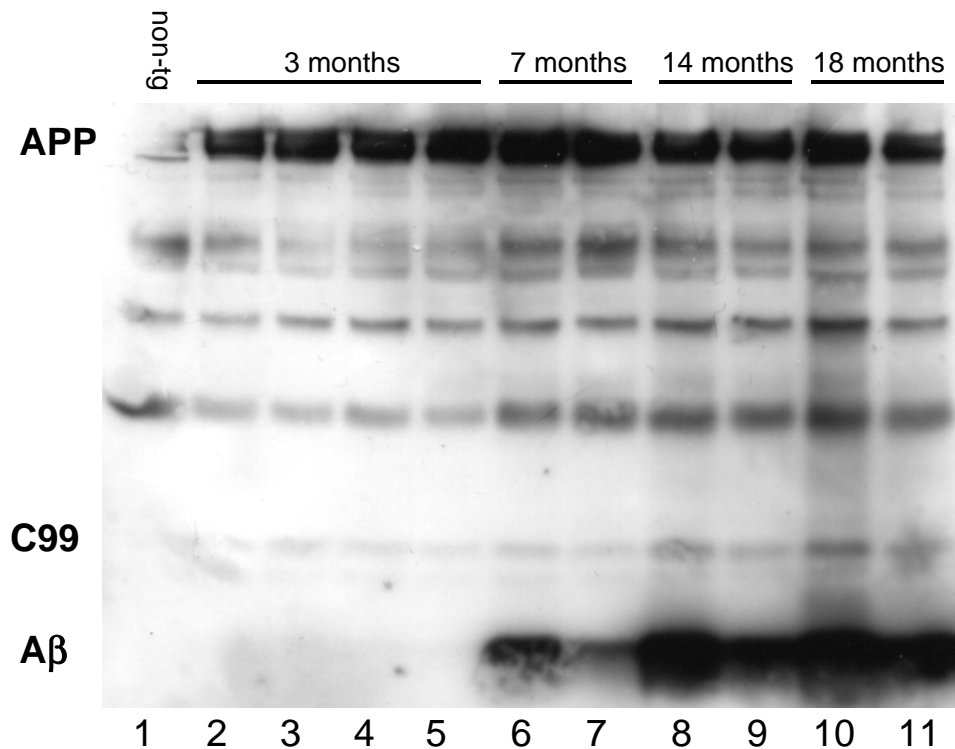


Figure 3.27: Effect of aging on accumulation of soluble A β in Thy1-APP transgenic mice.

Brains were lysed in Tris-buffered saline and blotted for analysis of APP expression and soluble A β levels. APP is detected at approximately 100 kDa, the C99 peptide at 12 kDa and the A β peptide at 4 kDa. Other bands are unspecific staining as can be seen from a non-transgenic control sample blotted on lane 1. The A β band is almost undetectable in 3 months old mice (lanes 2-5), but the intensity sharply increases up to an age of 18 months (lanes 10, 11). Inter-individual differences in the intensity of the A β band could be detected at 7 months (lanes 6, 7), 14 months (lanes 8, 9) and 18 months of age (lanes 10, 11).

PDGF-APP and double transgenic PDGF-APP/PS1 transgenic mice also showed an age-associated accumulation of A β , but levels of A β formation were much lower in these mouse models compared to age-matched Thy1-APP transgenic mice (Figure 3.28 a).

An age-related accumulation of A β in PDGF-APP, PDGF-APP/PS1 and Thy1-APP transgenic mouse models has also been obtained with SDS-soluble brain extracts representing the insoluble pool of A β (data not shown). It must however be noted that the intensity of insoluble A β bands in SDS brain lysates was always higher than that of

soluble A β bands in Tris-buffered brain lysates (Figure 3.28 b), suggesting that the largest pool of A β is present in an aggregated, insoluble state. Levels of A β in soluble as well as insoluble fractions are seemingly correlated, since the relative intensities were similar in Tris- as well as SDS-lysates (see Figure 3.28 b, samples from the same individual animal have been blotted in lanes 1 and 4, 2 and 5 as well as 3 and 6, respectively). Furthermore, some inter-individual variability in the intensity of A β bands was observed in 13-15 months old PDGF-APP/PS1 mice despite similar expression levels of APP.

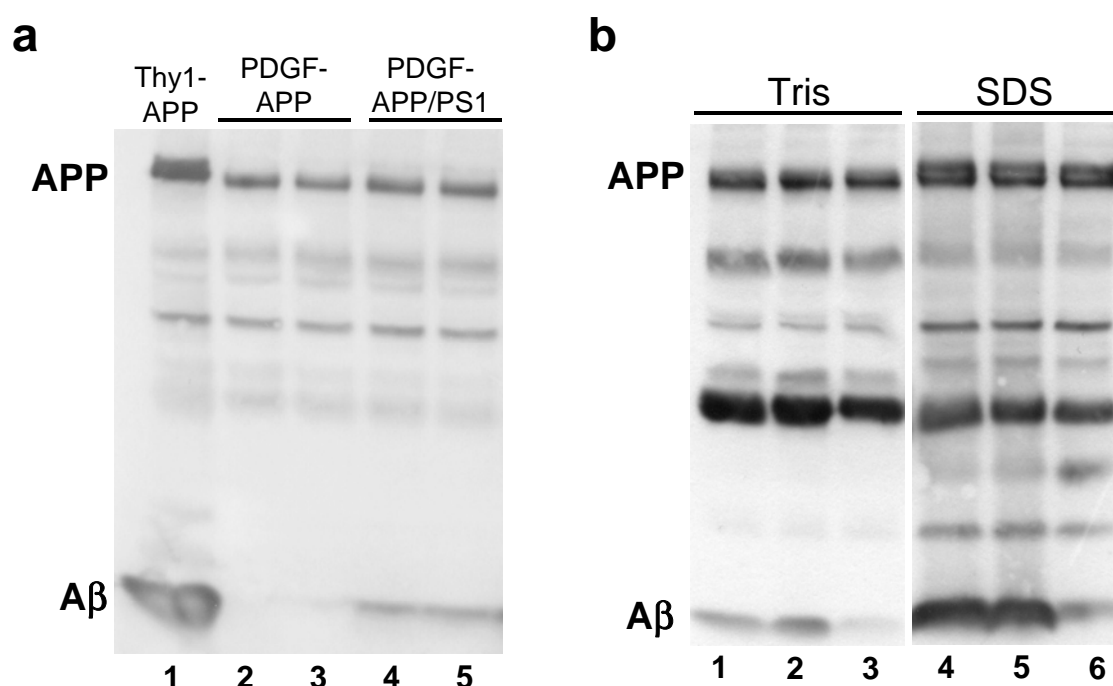


Figure 3.28: Levels of A β are higher in Thy1-APP compared to PDGF-APP and PDGF-APP/PS1 transgenic mice (a). The largest pool of A β is present in insoluble fractions (b).

a. Comparison of APP and soluble A β levels between Thy1-APP and PDGF-APP transgenic mice. Tris-lysates from 18 months old Thy1-APP transgenic (lane 1) or 19-22 months old PDGF-APP (lanes 2, 3) and 19-22 months old PDGF-APP/PS1 transgenic mice (lanes 4, 5) were blotted. The intensity of the A β band is highly increased in samples from the Thy1-APP transgenic mouse compared to the PDGF-APP single or double transgenics.

b. Differences between A β levels in Tris- and SDS-lysates from APP transgenic mouse brains. Brains from 13-15 months old PDGF-APP/PS1 transgenic mice were either lysed in Tris-buffered saline (lanes 1-3) or SDS-solution (lanes 4-6). The intensity of the A β band is much higher in SDS extracts. Lanes 1 and 4, 2 and 5 as well as 3 and 6 represent samples from the same animal. Levels of A β show some inter-individual variability in Tris-soluble as well as SDS extracts.

As can be seen in Figure 3.29, expression of full-length APP was only slightly higher in Thy1-APP compared to PDGF-APP and PDGF-APP/PS1 transgenic mice. However, the levels of C99 peptide were much higher in Thy1-APP transgenic mice, furthermore, levels of A β peptide were increased already in young Thy1-APP transgenic mice

RESULTS

(Figure 3.29, lanes 1-4). compared to middle-aged and aged single PDGF-APP transgenic mice (Figure 3.29, lanes 5,6,9,10). Coexpression of mutant PS1 in double transgenic PDGF-APP/PS1 mice led to higher formation of A β , but not C99 peptide (Figure 3.29, lanes 7,8,11,12) compared to single PDGF-APP transgenic mice. This is in good accordance with the anticipated effect, since mutant PS1 increases γ -secretase cleavage independently of β -secretase cleavage, leaving levels of C99 peptide derived from β -secretase cleavage unaffected. Again, PDGF-APP/PS1 transgenic mice at an age of 13-15 months showed rather large inter-individual variability in levels of A β (compare Figure 3.29, lanes 7 and 8).

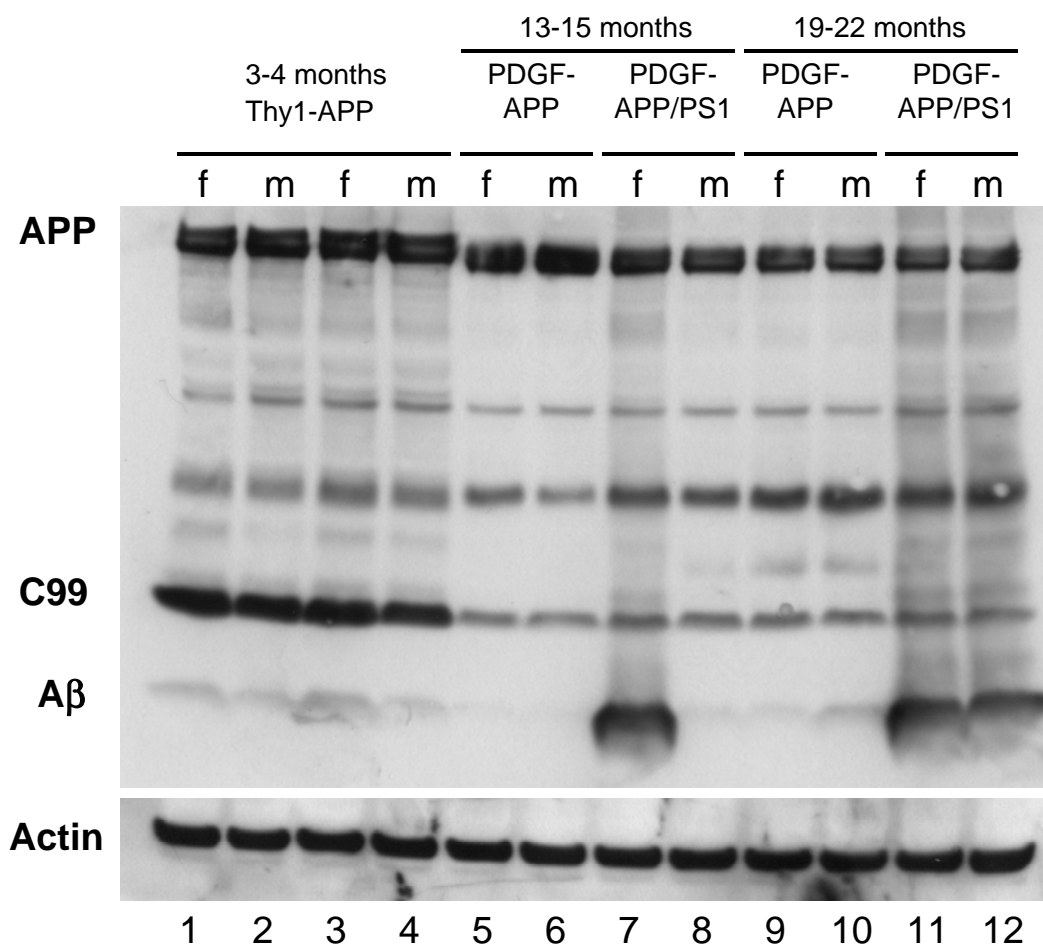


Figure 3.29: Expression of APP and insoluble A β levels in SDS-lysates from APP transgenic mouse brains.

Upper panel: SDS-buffer lysates of transgenic mouse brains were blotted for analysis of APP expression and insoluble A β levels. APP is detected at approximately 100 kDa. The longer 751 amino acid isoform in Thy1-APP transgenic mice (lanes 1-4) gives a protein band above that of the 695 isoform expressed in PDGF-APP transgenic mice (lanes 5-12). f = female, m = male mice.

Lower panel: Actin protein bands were detected as loading control.

In summary, the different APP transgenic mouse models used in this thesis express similar amounts of full-length APP. However, formation of the C99 peptide is highest in Thy1-APP transgenic mice, and similarly formation of A β decreases in the order of Thy1-APP > PDGF-APP/PS1 double transgenic mice > PDGF-APP single transgenic mice.

Furthermore, a gender difference in the intensity of the C99 peptide and insoluble A β bands was detected in 3 months old Thy1-APP transgenic mice (Figure 3.29, lanes 1-4). This was analyzed in more detail in a larger group of animals. Densitometric quantification of band intensity revealed that A β levels are approximately 30 % higher in female Thy1-APP transgenic mice at 3 months of age (Figure 3.30).

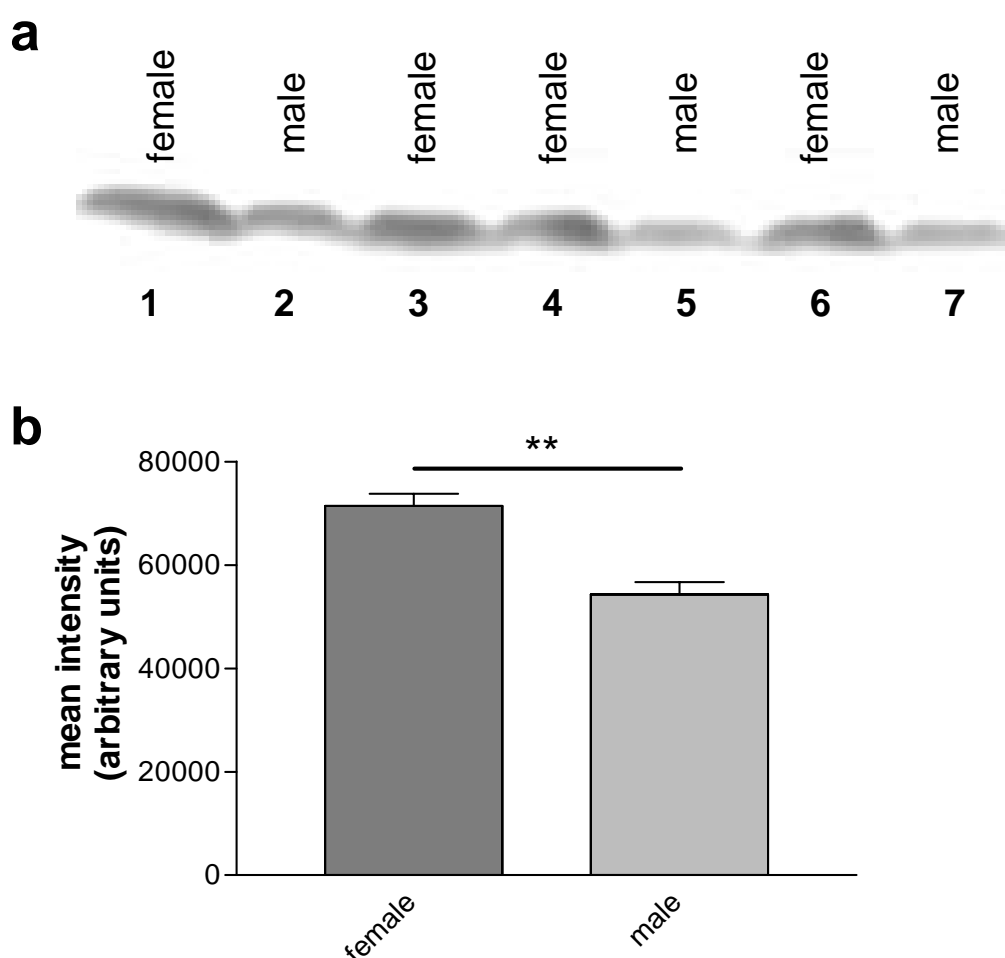


Figure 3.30: Gender differences in levels of insoluble A β in Thy1-APP transgenic mice.

a. A β protein band in Western blot of SDS brain lysates from 3 months old female and male Thy1-APP transgenic mice.

b. Densitometric evaluation of A β protein band shown in a. t-test ** $p < 0.01$.

3.2.4.2 Quantitative analysis of soluble A β ₁₋₄₀ levels in APP transgenic mice with ELISA

The results regarding the expression levels of A β obtained in Western blot analysis were further quantified with ELISA specific for human A β ₁₋₄₀. For this analysis, only Tris-buffered brain extracts representing the pool of soluble A β could be used.

As already observed in Western blots, an increase in the levels of A β with aging was present in both the PDGF-APP/PS1 and the Thy1-APP transgenic mouse models. As can be seen in Figure 3.31, levels of A β ₁₋₄₀ were almost undetectable in non-transgenic littermate control mice and were still at very low levels in single PDGF-APP transgenic mice. In these mice, no increase in A β ₁₋₄₀ with aging could be observed (Figure 3.31, left bars). In contrast, the additional expression of human mutant PS1 in double transgenic PDGF-APP/PS1 mice led to formation of much higher A β ₁₋₄₀ levels and accumulation during aging (Figure 3.31, right bars, note the different scales of axes).

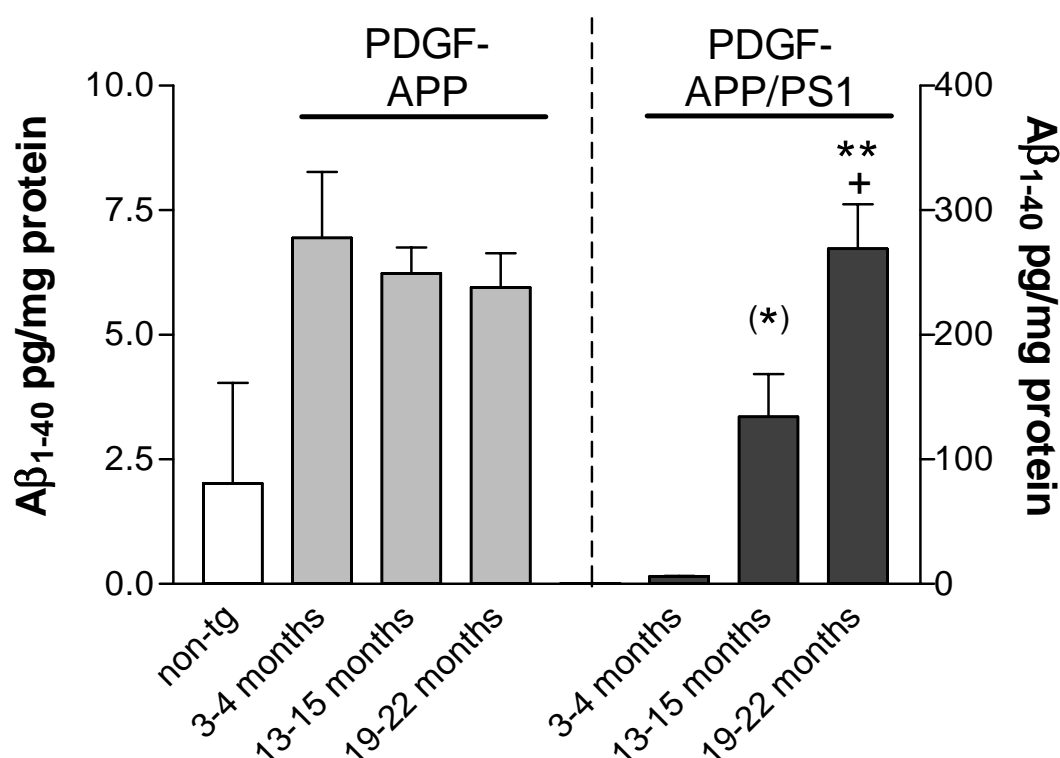


Figure 3.31: Levels of Aβ₁₋₄₀ in PDGF-APP and PDGF-APP/PS1 transgenic mice during aging.

Aβ levels were quantified with ELISA in Tris-buffered brain homogenates from non-transgenic littermate control mice (non-tg), PDGF-APP and PDGF-APP/PS1 transgenic mice at different ages. White bars: non-transgenic mice ($n = 2$), grey bars: single transgenic PDGF-APP mice (each age group $n = 2$), black bars: double transgenic PDGF-APP/PS1 mice (3-4 months $n = 2$; 13-15 months $n = 5$, 19-22 months $n = 6$). One-way ANOVA PDGF-APP/PS1 mice: $p < 0.01$ difference between age groups. Post hoc t -test: (*) $p < 0.1$ 13-15 months vs 3-4 months, ** $p < 0.01$ 19-22 months vs 3-4 months, + $p < 0.05$ 19-22 vs 13-15 months. Note the different scales of axes.

Levels of soluble Aβ₁₋₄₀ were however still higher in Thy1-APP transgenic mice and showed a marked accumulation with aging (Figure 3.32 a). Furthermore, the gender difference observed in insoluble total Aβ levels in Western blots was also found in soluble Aβ₁₋₄₀ levels quantified with ELISA (Figure 3.32 b). Of note, the relative gender difference in 3 months old animals in levels of soluble Aβ₁₋₄₀ quantified by ELISA is approximately 30% and very similar to the relative difference in levels of insoluble total Aβ quantified from densitometric evaluation of Western blot protein bands. This gender difference is still present in 12 months old animals, although absolute levels of Aβ₁₋₄₀ are much higher at that age.

In summary, the different APP transgenic mouse models display very different formation and accumulation of Aβ. While Aβ levels are very low and remain at this

RESULTS

level during aging in single PDGF-APP transgenic mice, double transgenic PDGF-APP/PS1 mice show increased A β levels and an age-associated accumulation. Highest levels of A β are observed in Thy1-APP transgenic mice. Relative to age-matched PDGF-APP/PS1 transgenic mice, levels of A β in Thy1-APP transgenic mice are 18-, 12- or 16-fold increased in 3-4, 12-15 or 18-22 months old mice, respectively.

Furthermore, a gender difference was observed in Thy1-APP transgenic mice, where female mice show higher formation of A β compared to male mice. No such gender difference was detected in PDGF-APP/PS1 transgenic mice (data not shown).

Of note, the gender difference in A β formation and accumulation during aging in Thy1-APP mice may be causally related to our observations on oxidative stress-related parameters, since (i) female Thy1-APP transgenic mice display oxidative damage and reduced activity of Cu/Zn-SOD earlier than male Thy1-APP mice and (ii) female 12 months old transgenic mice are more vulnerable towards oxidative *in vitro* stimulation of lipid peroxidation than male Thy1-APP mice.

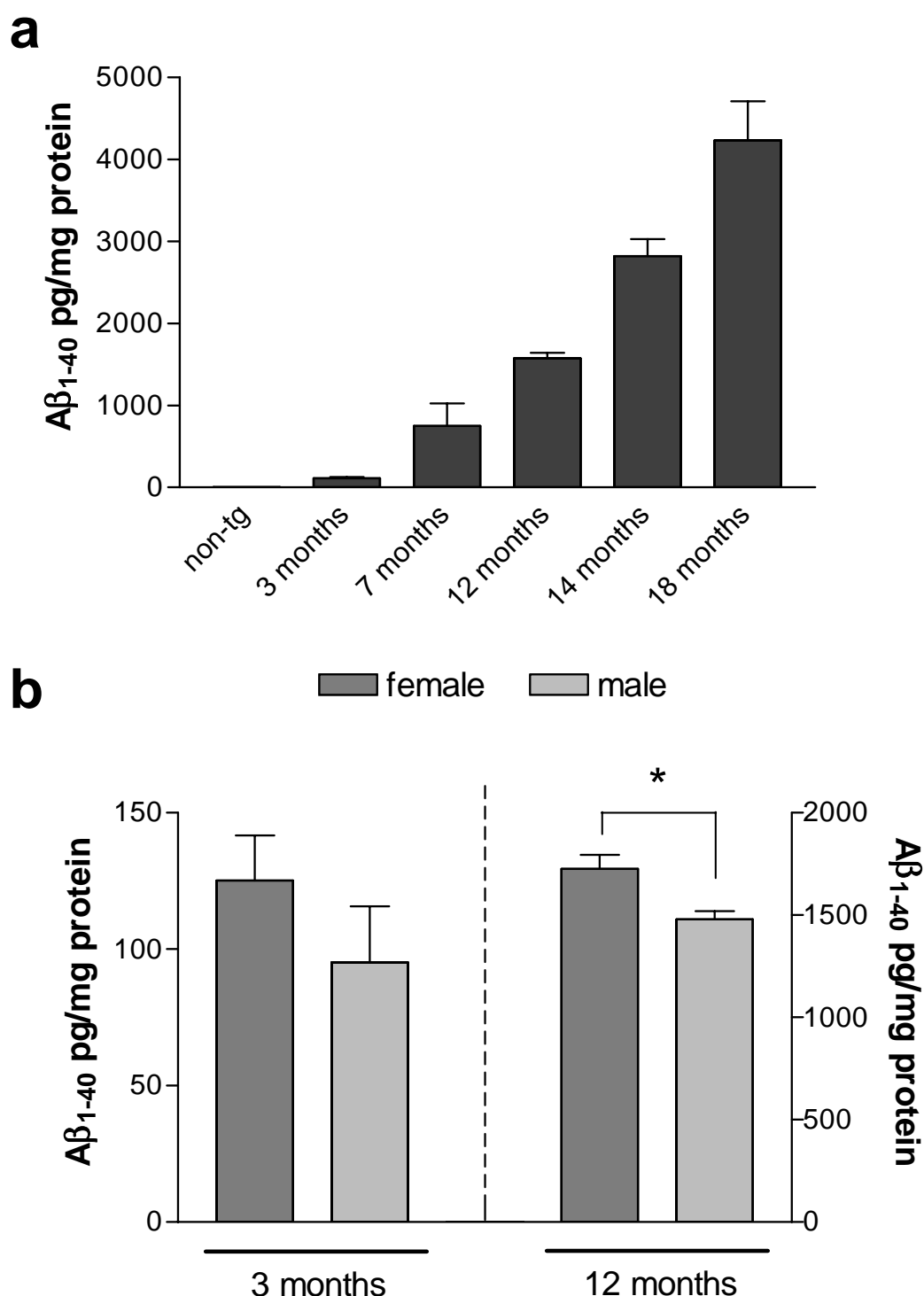


Figure 3.32: Accumulation of soluble $A\beta_{1-40}$ with age (a) and (b) gender difference in Thy1-APP transgenic mice.

$A\beta$ levels were quantified with ELISA in Tris-buffered brain homogenates from non-transgenic littermate control mice (non-tg) and Thy1-APP transgenic mice in different age groups as indicated.

a. Age-associated accumulation of soluble $A\beta$ in Thy1-APP transgenic mouse brains. $n = 2$ for each group except $n = 7$ for 3 months old and $n = 10$ for 12 months old Thy1-APP transgenic mice. One-way ANOVA $p < 0.0001$ effect of aging.

b. Gender difference in soluble $A\beta$ levels in brains from female and male Thy1-APP transgenic mice. 3 months old mice $n = 4$ female, $n = 3$ male, 12 months old mice $n = 6$ female, $n = 5$ male. Two-way ANOVA: $p < 0.05$ effect of gender, $p < 0.0001$ effect of age, interaction $p = 0.05$. Post hoc t-test: * $p < 0.05$ female vs male 12 months old mice. Note the different scales of axes.

3.3 ROS metabolism in brains from AD patients

Sporadic Alzheimer's disease patients represent the vast majority of AD cases, but the transgenic mouse models generated so far are all based on rare familial AD mutations and do not mimic the whole pathology of the disease in humans. Hence, we additionally studied frozen post mortem brain tissue from sporadic AD patients. Although post mortem tissue samples have limitations – as it is not possible to detect ROS directly and unknown confounding effects could be caused by the post mortem interval or tissue storage time – they nevertheless represent one of the best systems to study pathogenic events related to sporadic Alzheimer's disease. Brain tissue samples from sporadic AD patients and age-matched controls were analyzed for markers of lipid peroxidation products MDA and HNE, activities of antioxidant enzymes Cu/Zn-SOD, GPx and GR as well as MDA levels after stimulation of lipid peroxidation *in vitro*. Furthermore, in a subset of samples, levels of A β ₁₋₄₀ were determined to allow for a correlation with oxidative stress-related parameters.

3.3.1 Cohort #1

In a first study, a total number of 11 AD patients (7 female, 4 male) and 10 age-matched controls (6 female, 4 male) were studied. Brain tissue samples were obtained from 4 different brain regions: frontal cortex, parietal cortex, temporal cortex and cerebellum, which are differently affected by the disease in the order of temporal > frontal > parietal cortex (Delacourte et al., 1999), while cerebellar functions are not impaired by AD pathology. This allows for a broad screening of oxidative stress-induced alterations throughout various regions of the diseased brain.

3.3.1.1 Increased antioxidant metabolism in AD patients

Levels of lipid peroxidation products MDA and HNE were not significantly different between AD patients and controls (Figure 3.33 a and b, respectively). Levels of HNE showed however a tendency towards lower levels in cerebellum (Figure 3.33 b).

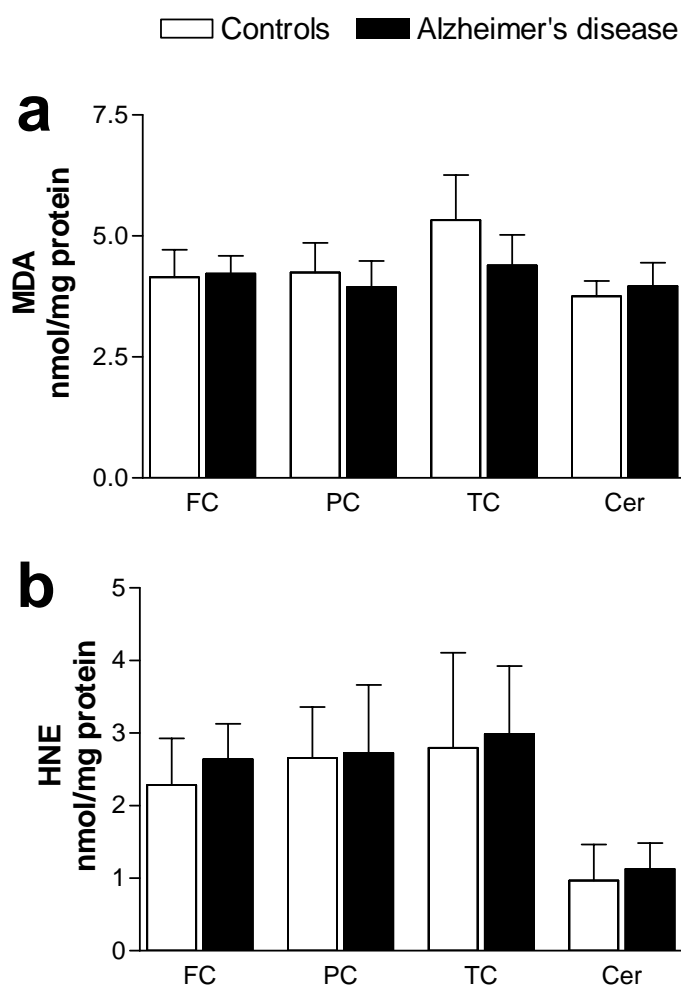


Figure 3.33: Levels of basal lipid peroxidation products MDA (a) and HNE (b) in AD brains.

Brain homogenates from frontal cortex (FC), parietal cortex (PC), temporal cortex (TC) and cerebellum (Cer) were analyzed for levels of lipid peroxidation products. $n = 8-10$ per group.

a. Basal MDA levels (nmol/mg protein). Two-way ANOVA: difference between groups *n.s.*

b. Basal HNE levels (nmol/mg protein). Two-way ANOVA: difference between groups *n.s.*, difference between brain regions $p = 0.08$.

Samples from AD patients showed significantly higher activities of Cu/Zn-SOD, GPx and GR (Figure 3.34). Thus, upregulation of antioxidant enzymes in AD patients may serve to inhibit increased formation of lipid peroxidation products. This upregulation is found in all brain regions analyzed, although to a different extent. In general, changes were most pronounced in samples from temporal cortex, which is a region severely affected by the disease (Detoledo-Morrell et al., 1997).

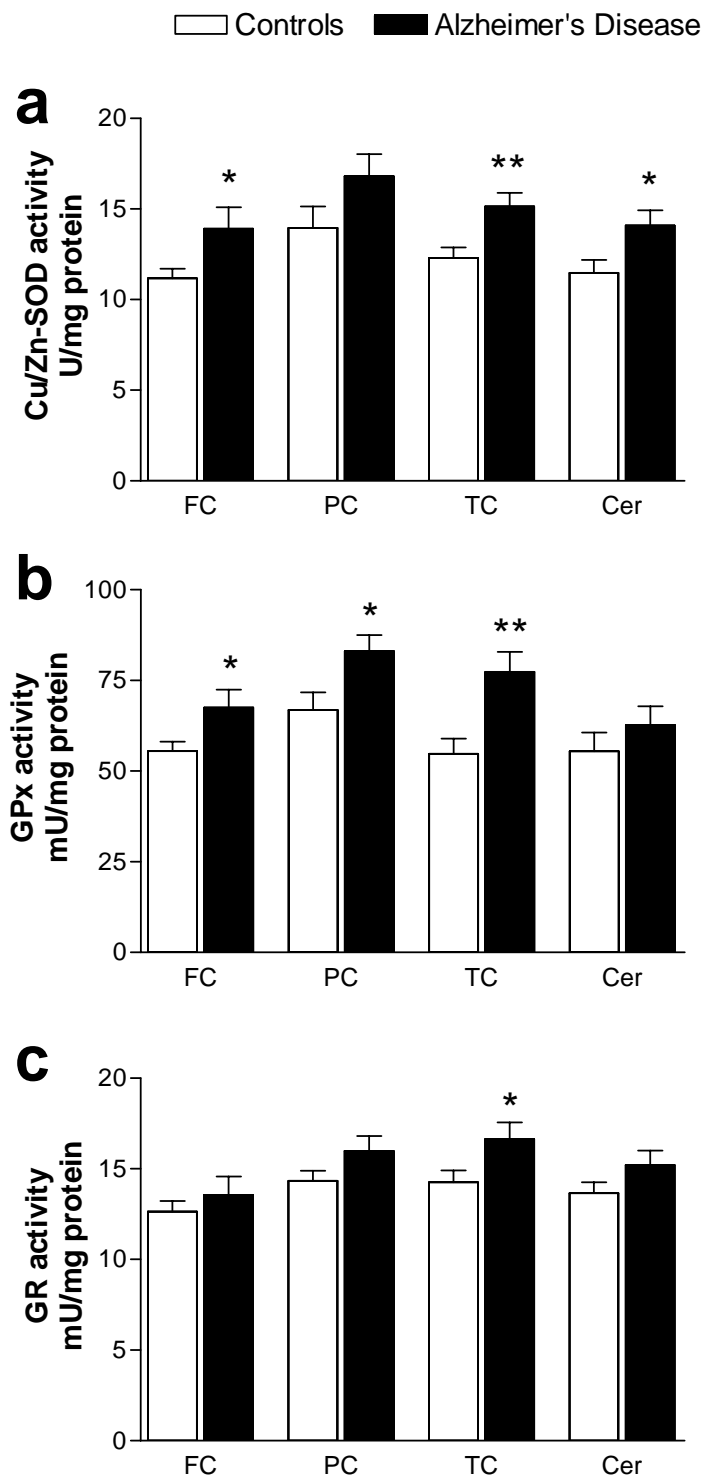


Figure 3.34: Increased activities of antioxidant enzymes in different brain regions from AD patients.

Brain homogenates from frontal cortex (FC), parietal cortex (PC), temporal cortex (TC) and cerebellum (Cer) were analyzed for activities of antioxidant enzymes. $n = 8-10$ per group.

a. Cu/Zn-SOD activity (U/mg protein). Two-way ANOVA: $p < 0.0001$ difference between groups. Post hoc t -test: * $p < 0.05$, ** $p < 0.01$ vs controls in the same brain region.

b. GPx activity (mU/mg protein). Two-way ANOVA: $p < 0.0001$ difference between groups. Post hoc t -test: * $p < 0.05$, ** $p < 0.01$ vs controls in the same brain region.

c. GR activity (mU/mg protein). Two-way ANOVA: $p < 0.05$ difference between groups. Post hoc t -test: * $p < 0.05$ vs controls in the same brain region.

Moreover, enzyme activities showed significant and positive correlations in the different brain regions, see the example of frontal cortex in Figure 3.35 and Table 3.6 for complete data. This indicates that not a single antioxidant enzyme is affected but rather that the whole cascade of antioxidant enzymatic defense is upregulated.

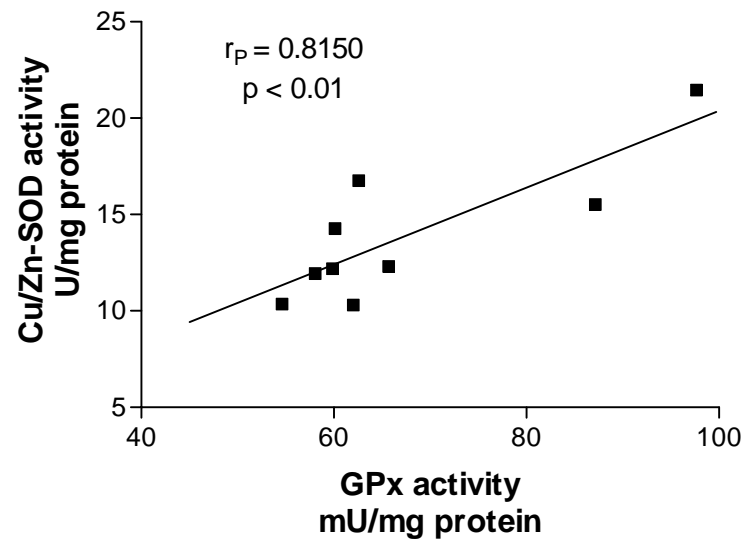


Figure 3.35: Correlation between Cu/Zn-SOD activity and GPx activity in samples from AD patients taken from frontal cortex.

Correlation: $r_P = 0.8150$, $p < 0.01$.

Table 3.6: Summary of correlations between different antioxidant enzyme activities in different brain regions.

Brain region	Group	Cu/Zn-SOD vs GPx	Cu/Zn-SOD vs GR	GPx vs GR
FC	All	$r_P = 0.8014$ ***	$r_P = 0.6465$ **	$r_P = 0.7639$ ***
	Control	$r_P = 0.5154$ n.s.	$r_P = 0.2584$ n.s.	$r_P = 0.5481$ (*)
	AD	$r_P = 0.8150$ **	$r_P = 0.7721$ *	$r_P = 0.8693$ **
PC	All	$r_P = 0.5768$ *	$r_P = 0.5899$ *	$r_P = 0.7442$ ***
	Control	$r_P = 0.1299$ n.s.	$r_P = -0.1118$ n.s.	$r_P = 0.8228$ *
	AD	$r_P = 0.7515$ *	$r_P = 0.8423$ **	$r_P = 0.6457$ (*)
TC	All	$r_P = 0.7378$ ***	$r_P = 0.6590$ **	$r_P = 0.6872$ **
	Control	$r_P = 0.3323$ n.s.	$r_P = 0.4524$ n.s.	$r_P = 0.6885$ *
	AD	$r_P = 0.7180$ *	$r_P = 0.5790$ n.s.	$r_P = 0.5073$ n.s.
Cer	All	$r_P = 0.8883$ ***	$r_P = 0.7648$ ***	$r_P = 0.6917$ ***
	Control	$r_P = 0.9580$ ***	$r_P = 0.5598$ (*)	$r_P = 0.6243$ *
	AD	$r_P = 0.8832$ ***	$r_P = 0.8179$ **	$r_P = 0.7753$ **

Asterisks indicate significance of correlation. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, (*) $p < 0.1$, n.s. not significant.

RESULTS

After *in vitro* stimulation, levels of MDA formation were significantly lower in AD patients (Figure 3.36), suggesting that the higher antioxidant enzyme activities in AD patients protect against oxidative stimuli.

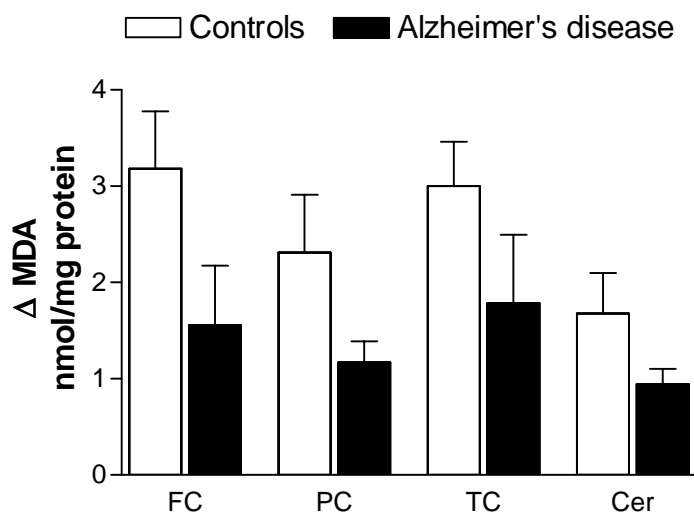


Figure 3.36: Stimulated MDA levels are reduced in AD brains.

Formation of MDA (nmol/mg protein) was stimulated with 100 μ M FeCl_3 for 30 min at 37°C. Brain homogenates from frontal cortex (FC), parietal cortex (PC), temporal cortex (TC) and cerebellum (Cer) were analyzed. $n = 8-10$ per group. Two-way ANOVA: $p < 0.01$ difference between groups.

3.3.1.2 Effect of gender on antioxidant metabolism in AD patients

Based on epidemiological reports of a higher risk for AD in females, we additionally analyzed the subgroups of each male and female patients and controls for a putative gender difference in oxidative stress parameters.

Interestingly, levels of HNE were higher in female compared to male AD patients (Figure 3.37).

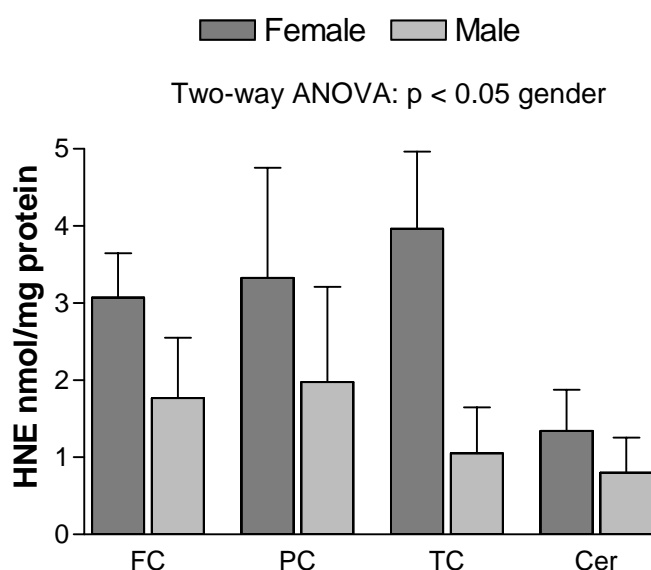


Figure 3.37: Gender difference in HNE levels among AD patients.

AD brain samples from frontal cortex (FC), parietal cortex (PC), temporal cortex (TC) and cerebellum (Cer) were analyzed for HNE levels (nmol/mg protein). $n = 4-6$. Two-way ANOVA: $p < 0.05$ difference between gender.

Furthermore, upregulation of activities of Cu/Zn-SOD and GPx was more pronounced in female compared to male AD patients when comparing all brain regions (Figure 3.38 a and b, respectively), while activity of GR was not different between the gender (Figure 3.38 c). No gender differences were found among male and female controls (data not shown).

These results suggest that the AD-related upregulation of antioxidant defense is more pronounced in female compared to male AD patients, while female patients at the same time display higher oxidative damage at the level of HNE. Thus, female patients are seemingly more vulnerable towards oxidative damage despite higher activities of Cu/Zn-SOD and GPx.

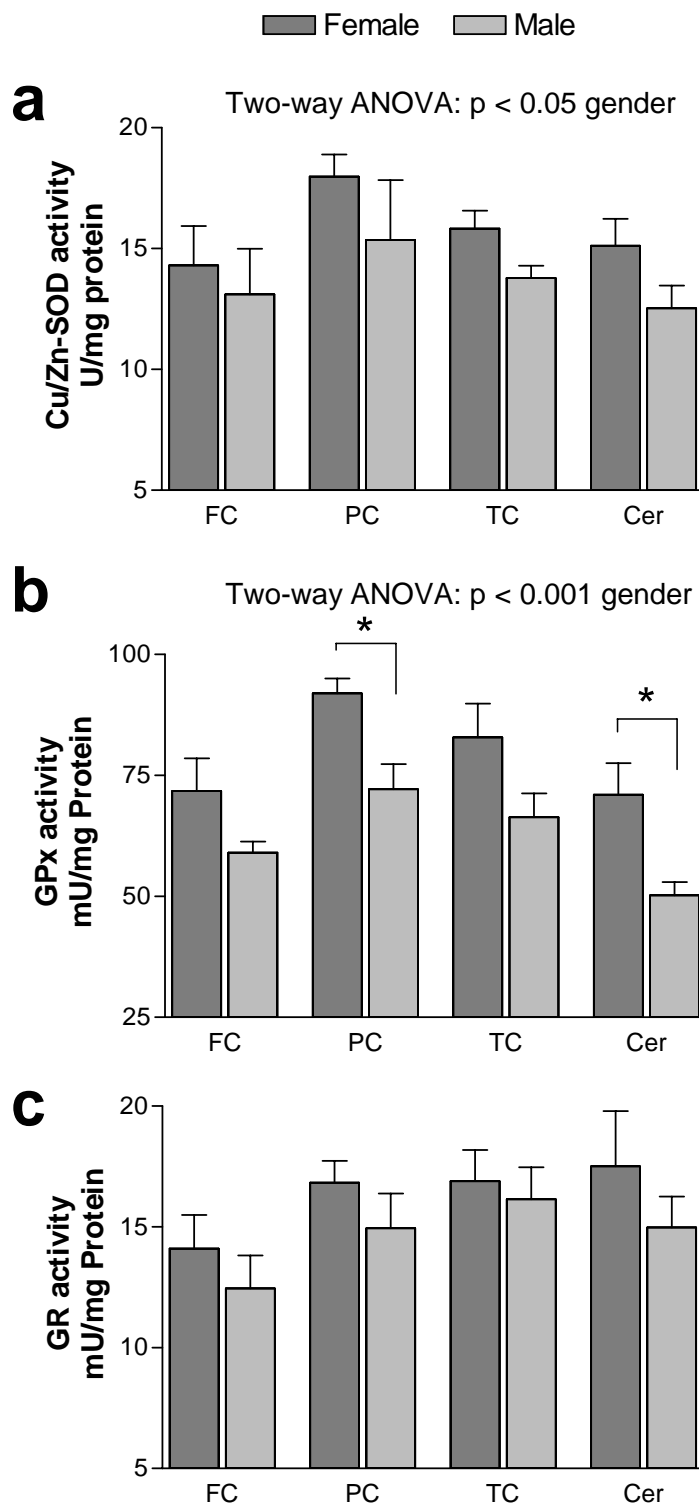


Figure 3.38: Gender differences in antioxidant enzyme activities among AD patients.

AD brain samples from frontal cortex (FC), parietal cortex (PC), temporal cortex (TC) and cerebellum (Cer) were analyzed for activities of antioxidant enzymes. $n = 4-6$.

a. Cu/Zn-SOD activity (U/mg protein). Two-way ANOVA: $p < 0.05$ difference between gender.

b. GPx activity (mU/mg protein). Two-way ANOVA: $p < 0.001$ difference between gender. Post hoc t-test * $p < 0.05$.

c. GR activity (mU/mg protein). Two-way ANOVA: difference between gender n.s.

3.3.2 Cohort #2

In a second study, only tissue samples from temporal cortex were analyzed, since the changes in oxidative stress parameters observed in cohort #1 were most pronounced in samples from temporal cortex, which is a severely affected brain region in AD. Tissue samples from a total of 14 controls (6 female, 8 male) and 16 sporadic AD patients (8 female, 8 male) were studied. Clinical and neuropathological data as well as Apo E genotype were available for most of the tissue samples, allowing for correlations of the biochemical parameters with clinical and neuropathological findings and Apo E genotype in the individual AD patient or control.

3.3.2.1 Changes in oxidative stress parameters in AD patients

As already observed in cohort #1, basal levels of MDA were not significantly different between AD patients and controls (Figure 3.39 a), although they were increased in AD patients. Levels of HNE were however significantly increased (Figure 3.39 b).

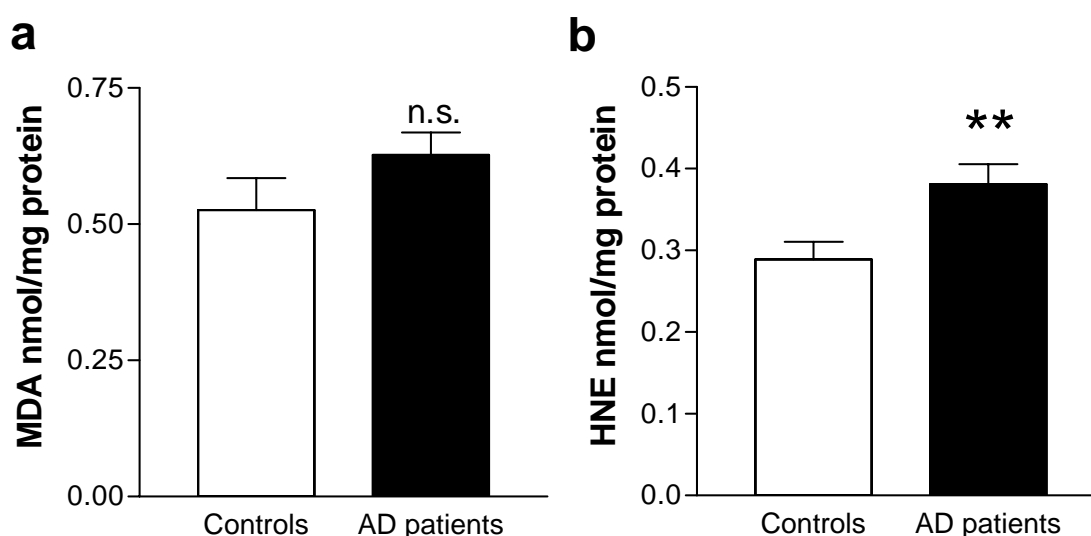


Figure 3.39: Basal levels of lipid peroxidation products in AD patients.

Brain homogenates from temporal cortex were analyzed for basal levels of MDA (a) and HNE (b). $n = 14$ for controls and $n = 16$ for AD patients.

a. Basal MDA levels (nmol/mg protein). t -test $n.s.$

b. Basal HNE levels (nmol/mg protein). t -test $p < 0.01$ AD patients vs controls.

In good accordance with cohort #1, we found increased activities of antioxidant enzymes in brain samples from AD patients. However, the relative increase was not as pronounced as in cohort #1 and only significant for Cu/Zn-SOD and GPx, but not for GR activity (Figure 3.40 a, b and c, respectively). Again, enzyme activities were positively and significantly correlated in subgroups of AD patients and controls (see

RESULTS

Table 3.7), confirming that the whole cascade of antioxidant enzymes is affected as already observed previously in cohort #1.

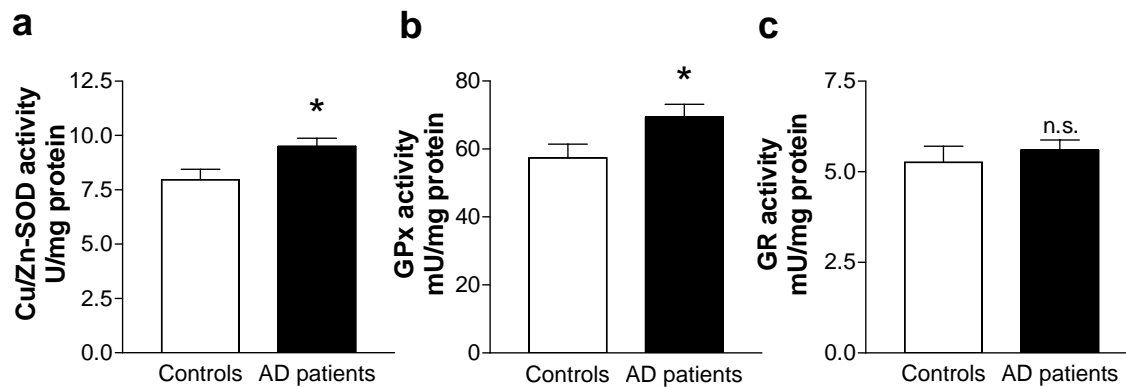


Figure 3.40: Increased activities of Cu/Zn-SOD and GPx in AD patients.

Brain homogenates from temporal cortex were analyzed for activities of antioxidant enzymes. $n = 14$ for controls and $n = 16$ for AD patients.

a. Cu/Zn-SOD activity (U/mg protein). t -test * $p < 0.05$ controls vs AD patients.

b. GPx activity (mU/mg protein). t -test * $p < 0.05$ controls vs AD patients.

c. GR activity (mU/mg protein). t -test n.s.

Table 3.7: Summary of correlations between different antioxidant enzyme activities in cohort #2.

Group	Cu/Zn-SOD vs GPx	Cu/Zn-SOD vs GR	GPx vs GR
All	$r_P = 0.9068$ ***	$r_P = 0.7312$ ***	$r_P = 0.7683$ ***
Control	$r_P = 0.8741$ ***	$r_P = 0.7783$ ***	$r_P = 0.8819$ ***
AD	$r_P = 0.8777$ ***	$r_P = 0.7348$ ***	$r_P = 0.7031$ ***

Asterisks indicate significance of correlations. *** $p < 0.001$.

Higher levels of MDA were correlated with increased activities of each Cu/Zn-SOD, GPx and GR activity (see Table 3.8). Similar correlations were found for HNE levels, which was however only statistically significant for correlation with Cu/Zn-SOD but not GPx or GR activity (see Table 3.8 for complete data and Figure 3.41 as example). These results suggest that oxidative damage is accompanied by increased antioxidant defenses.

Table 3.8: Correlations between different antioxidant enzyme activities and levels of lipid peroxidation products MDA or HNE.

aldehyde vs enzyme	Cu/Zn-SOD	GPx	GR
MDA	$r_P = 0.6728$ $p < 0.0001$	$r_P = 0.6525$ $p < 0.0001$	$r_P = 0.5109$ $p < 0.01$
HNE	$r_P = 0.3384$ $p < 0.05$	n.s.	n.s.

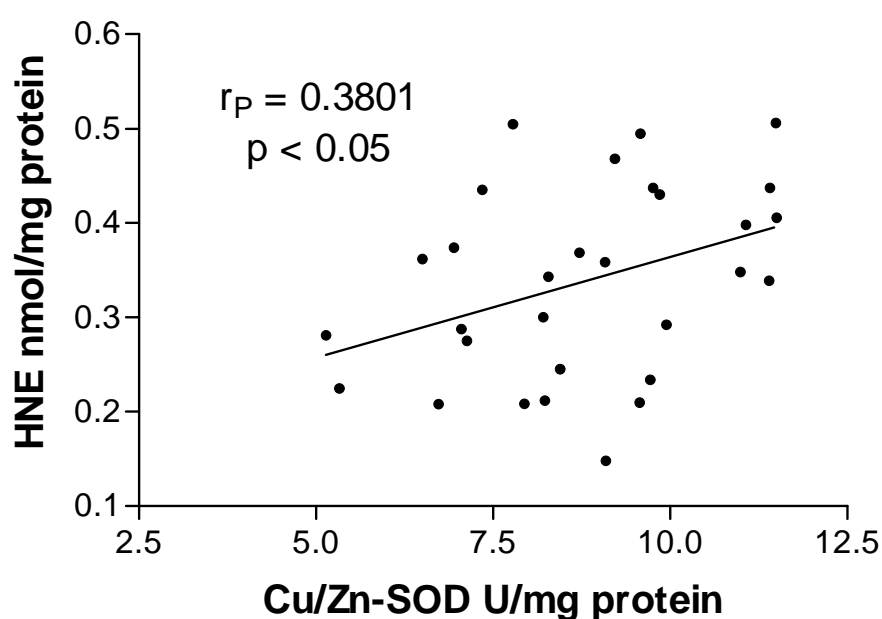


Figure 3.41: Correlation between HNE levels and Cu/Zn-SOD activity.

HNE levels (nmol/mg protein) were plotted against Cu/Zn-SOD activity (U/mg protein) in samples from controls and AD patients. Correlation. $r_P = 0.3801$, $p < 0.05$.

Stimulation of lipid peroxidation led to increased formation of MDA in samples from AD patients (Figure 3.42 a). However, in both the control and AD group, the effect of stimulation showed a large variability due to bimodal distribution of values: in both groups, local clusters of values well below and above 4 nmol/mg protein MDA formation after stimulation could be identified (Figure 3.42 b).

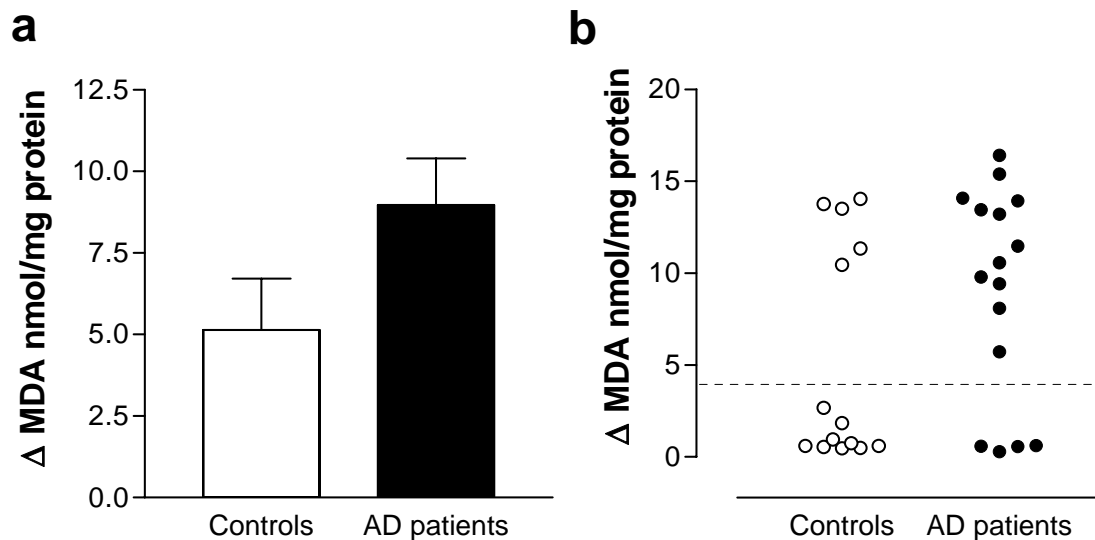


Figure 3.42: Stimulated levels of MDA in AD patients.

Brain homogenates taken from temporal cortex were analyzed for levels of MDA after stimulation with 100 μM FeCl_3 for 30 min at 37°C. $n = 14$ for controls and $n = 16$ for AD patients.

a. Bar graph.

b. Scatterplot. In both control and AD group, samples that are resistant to stimulation – with stimulated MDA levels below an arbitrary threshold value of 4 nmol/mg protein – as well as samples that can be stimulated – with levels above 4 nmol/mg protein – can be identified. In the control group, only 5 of 14 individual samples representing 36 % of subjects could be stimulated, whereas in the AD group, 12 of 16 individual samples representing 75 % could be stimulated.

Due to this bimodal distribution, the t-test assuming Gaussian distribution is not the appropriate statistical instrument to use. Therefore, chi-squared test was calculated. If “responders” are defined by more than 4 nmol/mg protein MDA formation after stimulation, 5 of a total of 14 subjects in the control group representing 36 % compared to 12 of a total of 16 subjects in the AD group representing 75 % can be classified as “responders” (Table 3.9). Calculating the chi-squared value for this distribution gives $\chi^2 = 4.69$, which is above the critical value of 3.841 for the 5 % limit of significance, indicating a significant difference between controls and AD patients ($p < 0.05$).

Table 3.9: Distribution of samples from non-demented controls and sporadic AD patients classified as “responders” or “non-responders” according to the extent of MDA formation after stimulation with ferric iron.

	“non-responder”	“responder”	Total
Controls	9	5	14
AD patients	4	12	16
Total	13	17	30

“Responder” were classified as samples that exhibit more than 4 nmol/mg protein elevated MDA levels after stimulation of brain homogenates with 100 μM ferric iron for 30 min at 37°C. “Non-responder” represent samples where less than 4 nmol/mg protein MDA after stimulation were formed. $\chi^2 = 4.69$, $p < 0.05$ significant difference between controls and AD patients.

The factors that contribute to the MDA response after *in vitro* stimulation could not be elucidated. The groups of each responders and non-responders were not different in age, gender, post mortem interval, Apo E genotype or activities of antioxidant enzymes. Also, the tissue storage time of samples, the cause of death and the neuropathological Braak staging, the disease duration or the MMSE score gave no clear indication of differences between responders and non-responders. Thus, either unknown factors or a combination of several singular factors, which can not be statistically elucidated in our rather small group with 30 subjects, may affect the outcome of MDA formation after *in vitro* stimulation.

In contrast to cohort #1, no gender differences between male and female AD patients in the activities of antioxidant enzymes or lipid peroxidation products were found in this study (data not shown).

3.3.2.2 Levels of soluble A β ₁₋₄₀ in brains from AD patients – correlation with Apo E4 genotype

Since the primary toxicity mediating the development of Alzheimer's disease is hypothetically attributed to the A β peptide, levels of soluble A β ₁₋₄₀ were analyzed in a subgroup of 15 samples (7 controls, 8 AD patients). The mean A β ₁₋₄₀ concentration was significantly higher in AD patients compared to controls (Figure 3.43 a). A large overlap between the two groups exists, and few individual samples showed very high A β ₁₋₄₀ concentrations with more than 250 pg/ml in the control group and more than 750 pg/ml in the AD group (Figure 3.43 b).

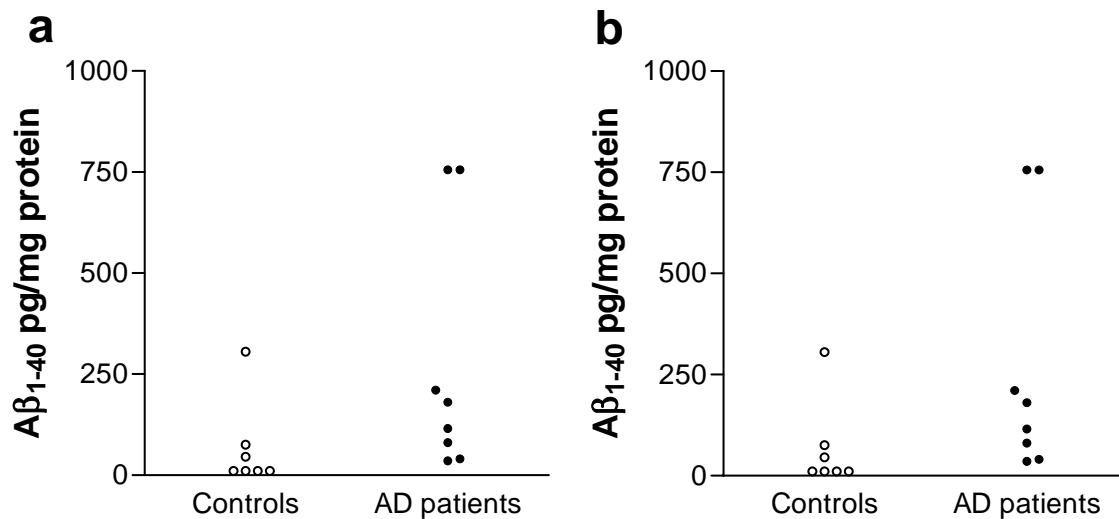


Figure 3.43: Levels of soluble $A\beta_{1-40}$ in AD patients and controls.

$A\beta_{1-40}$ levels (pg/mg protein) were quantified from Tris-soluble brain tissue homogenates with an ELISA specific for $A\beta_{1-40}$. $n = 7$ for controls, $n = 8$ for AD patients.

a. Bar graph. * $p < 0.05$ controls vs AD patients (Mann-Whitney-test).

b. Scatterplot. Some overlap between groups exists, furthermore few samples contain very high $A\beta_{1-40}$ levels.

When analyzing subgroups of controls and AD patients according to the presence of Apo E4 alleles, the levels of $A\beta_{1-40}$ were strongly elevated in Apo E4 homozygous carriers compared to heterozygous E4 and non-E4 carriers (Figure 3.44 a). Furthermore, a protective effect of Apo E2 alleles against $A\beta_{1-40}$ elevation is observed when the exact Apo E genotype is analyzed (Figure 3.44 b), although statistical evaluation was precluded due to small numbers of subjects in the respective subgroups. Nevertheless, despite the fact that the number of homozygous Apo E4 carriers consisted of only 2 AD patients, the small variation in values argues for a prominent role of Apo E4 in formation and/or accumulation of $A\beta$ in these subjects. Of note, the Apo E4 allele is the most important genetic risk factor for sporadic AD identified so far.

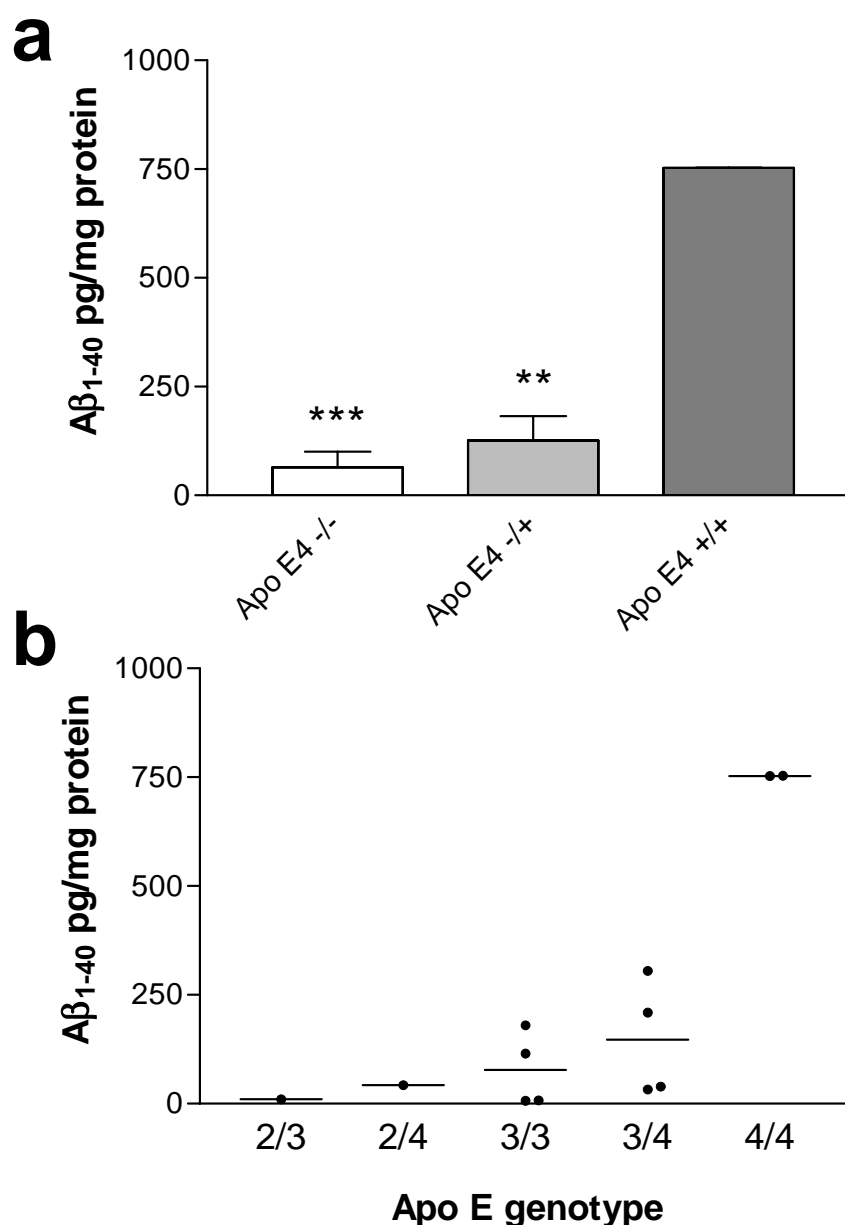


Figure 3.44: Aβ₁₋₄₀ levels depend on Apo E4 genotype.

Aβ₁₋₄₀ levels (pg/mg protein) were quantified from Tris-soluble brain tissue homogenates with an ELISA specific for Aβ₁₋₄₀. Results were analyzed according to Apo E4 genotype:

a. Subjects were grouped according to the presence of Apo E4 alleles. $n = 5$ subjects without Apo E4 alleles (3 controls, 2 AD patients, Apo E4 -/-), $n = 5$ heterozygous Apo E4 subjects (2 controls, 3 AD patients, Apo E4 +/-) and $n = 2$ homozygous Apo E4 carriers (2 AD patients, Apo E4 +/+) were analyzed. Aβ₁₋₄₀ levels are strongly elevated in Apo E4 carriers. One-way ANOVA $p < 0.0001$. Post hoc t-test *** $p < 0.001$ Apo E4 -/- vs Apo E4 +/+, ** $p < 0.01$ Apo E4 +/- vs Apo E4 +/.

b. Subjects were grouped according to the exact Apo E genotype.

3.3.2.3 Correlations of lipid peroxidation products and antioxidant enzyme activities with levels of soluble A β_{1-40}

Levels of A β_{1-40} showed a positive correlation with MDA levels, which just failed to reach statistical significance ($p = 0.05$, Figure 3.45). A β_{1-40} levels did not correlate with HNE levels or stimulated MDA levels (data not shown), but were positively correlated with activities of Cu/Zn-SOD and GPx (Figure 3.46 a and b, respectively) and showed a trend towards correlation with GR ($r_s = 0.4643$, $p = 0.08$, data not shown). These results suggest that increased A β_{1-40} levels promote oxidative damage and at the same time cause upregulation of activities of Cu/Zn-SOD and GPx.

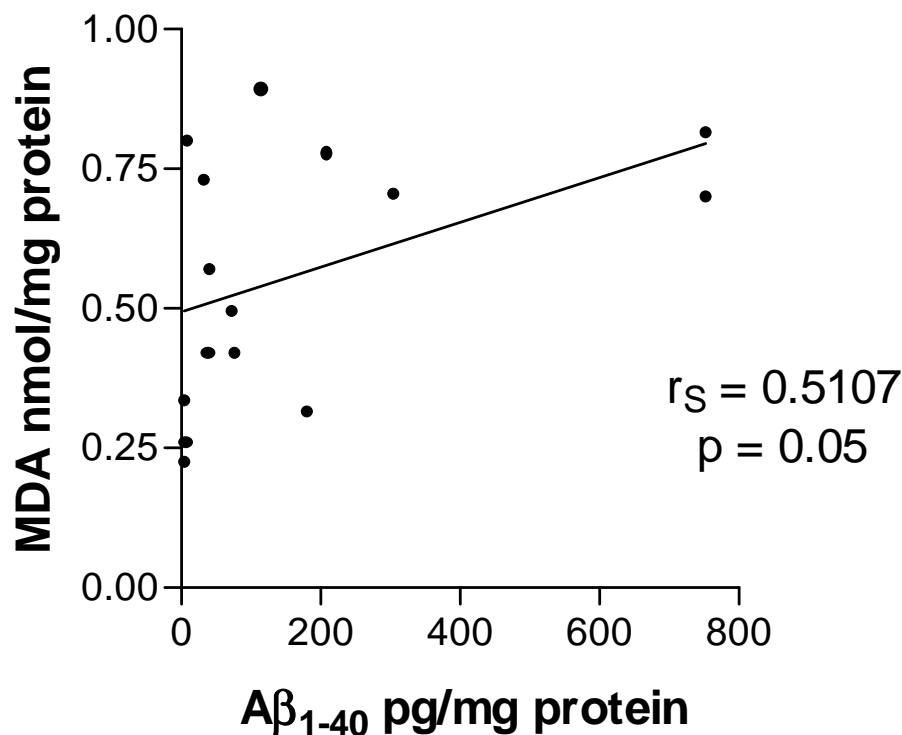


Figure 3.45: Correlation between MDA and A β_{1-40} levels in controls and AD patients.

MDA levels (nmol/mg protein) were plotted against A β_{1-40} levels (pg/mg protein) in samples from controls and AD patients. Correlation: $r_s = 0.5107$, $p = 0.05$.

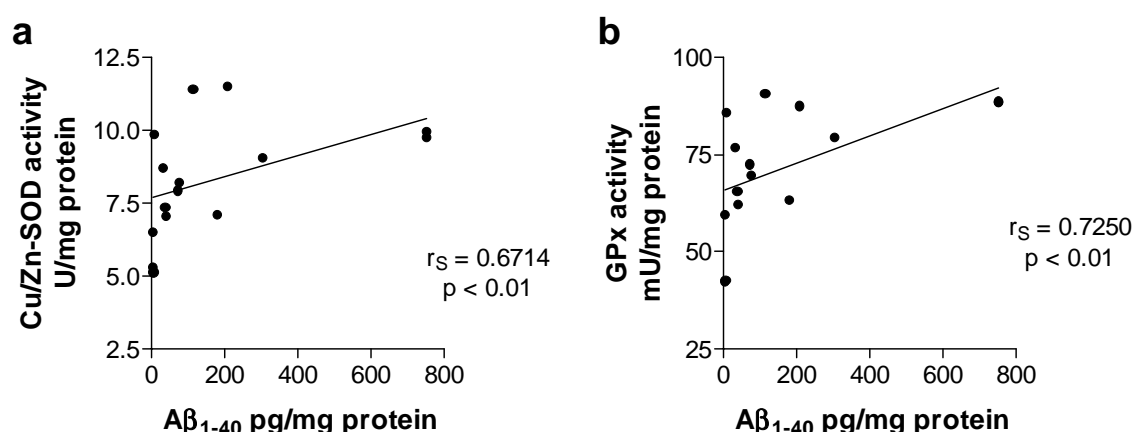


Figure 3.46: Correlation between antioxidant enzymes Cu/Zn-SOD (a) or GPx (b) and $A\beta_{1-40}$ levels in controls and AD patients.

a. Cu/Zn-SOD activity (U/mg protein) was plotted against $A\beta_{1-40}$ levels (pg/mg protein) in samples from controls and AD patients. Correlation: $r_s = 0.6714$, $p < 0.01$.

b. GPx activity (mU/mg protein) was plotted against $A\beta_{1-40}$ levels (pg/mg protein) in samples from controls and AD patients. Correlation: $r_s = 0.7250$, $p < 0.01$.

3.3.2.4 Correlations of lipid peroxidation products and antioxidant enzyme activities with Apo E genotype

Since the Apo E4 allele is the second most important risk factor – apart from aging – for sporadic AD, we speculated whether oxidative damage and changes in antioxidant defense may be related to the presence of one or two Apo E4 alleles.

Levels of lipid peroxidation products MDA and HNE were elevated depending on the number of Apo E4 alleles (Figure 3.47). Differences were not significant for MDA, but showed a trend for HNE (One-way ANOVA $p = 0.077$).

Activities of antioxidant enzymes were similarly elevated in homozygous Apo E4 carriers (Figure 3.48), but this effect was not statistically significant for any of the antioxidant enzymes analyzed.

These results suggest that Apo E4 alleles favour the formation of lipid peroxidation products. However, due to the lack of controls homozygous for Apo E4 this effect could also be inherently related to the fact that AD patients and controls were not distributed equally between the three groups. We therefore additionally evaluated only AD patients and controls separately and similar results were obtained, although this proved to be not statistically significant due to smaller numbers of subjects. Nevertheless, levels of HNE were still elevated in AD patients compared to controls in each the groups of non-E4 carriers and heterozygous E4 carriers (Figure 3.49).

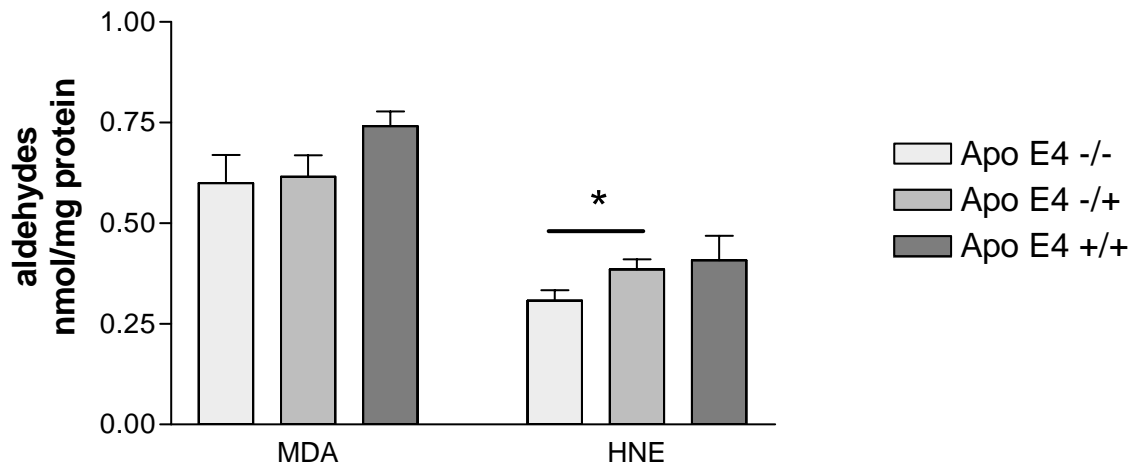


Figure 3.47: Levels of lipid peroxidation products MDA and HNE in relation to Apo E4 genotype.

Levels of lipid peroxidation products MDA and HNE (nmol/mg protein) were analyzed according to Apo E4 genotype. $n = 11$ subjects without Apo E4 alleles (6 controls, 5 AD patients, Apo E4 -/-), $n = 9$ heterozygous Apo E4 subjects (3 controls, 6 AD patients, Apo E4 -/+) and $n = 3$ homozygous Apo E4 carriers (3 AD patients, Apo E4 +/+) were analyzed.

MDA: One-way ANOVA n.s.

HNE: One-way ANOVA: $p = 0.077$. Post hoc t-test * $p < 0.05$ Apo E4 -/- vs Apo E4 -/+.

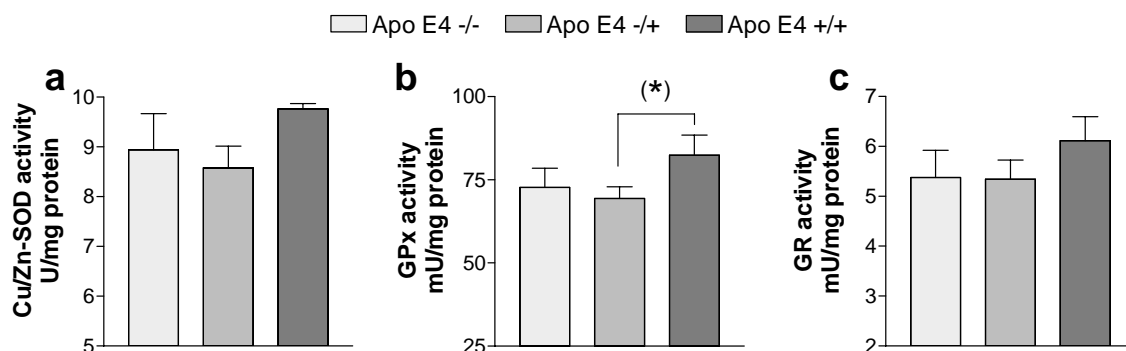


Figure 3.48: Activities of antioxidant enzymes in relation to Apo E4 genotype.

Activities of antioxidant enzymes Cu/Zn-SOD (a), GPx (b) and GR (c) were analyzed according to Apo E4 genotype. $n = 11$ subjects carrying no Apo E4 alleles (6 controls, 5 AD patients, Apo E4 -/-), $n = 9$ heterozygous Apo E4 subjects (3 controls, 6 AD patients, Apo E4 -/+) and $n = 3$ homozygous Apo E4 carriers (3 AD patients, Apo E4 +/+) were analyzed. Differences are not statistically significant in One-way ANOVA. Post hoc t-test (*) $p = 0.09$ GPx activity Apo E4 -/+ vs Apo E4 +/+.

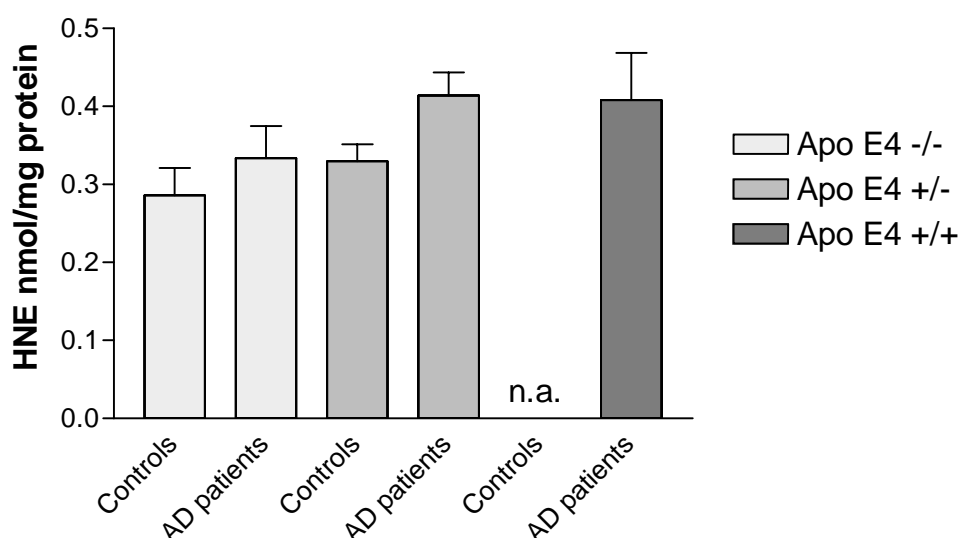


Figure 3.49: HNE levels in controls and AD patients in relation to Apo E4 genotype.

HNE levels were analyzed according to Apo E genotype in control and AD group separately. Apo E4 homozygous controls were not available (n.a.). $n = 6$ controls Apo E4 -/-, $n = 5$ AD patients Apo E4 -/-, $n = 3$ controls Apo E4 +/-, $n = 6$ AD patients Apo E4 +/-, $n = 3$ AD patients Apo E4 +/+. One-way ANOVA $p = 0.10$. Differences between controls and AD patients in the same Apo E genotype group are not statistically significant.

3.3.2.5 Correlations of lipid peroxidation products and antioxidant enzyme activities with mini mental status (MMSE) score

MMSE (mini mental status examination) data were available for 2 controls and 11 AD patients. The MMSE test is widely used as a routine measure of cognitive abilities in demented patients. Non-demented individuals can score a maximum of 30 points – depending on the age and degree of school education – whereas demented patients usually have much lower values. In our sample, two control individuals scored 29 and 28 points indicating no cognitive impairment, whereas the AD patients scored 21 or less points indicating mild to severe cognitive impairment. One patient was even unable to conduct the test at all, scoring 0 points.

We speculated whether the degree of cognitive impairment quantified with MMSE was related to increased oxidative damage as measured by the levels of lipid peroxidation products. Interestingly, levels of MDA were negatively correlated with the MMSE score, which just failed to reach statistical significance ($p = 0.055$, Figure 3.50). This indicates that oxidative damage at the level of MDA increases during the clinical progression of the disease.

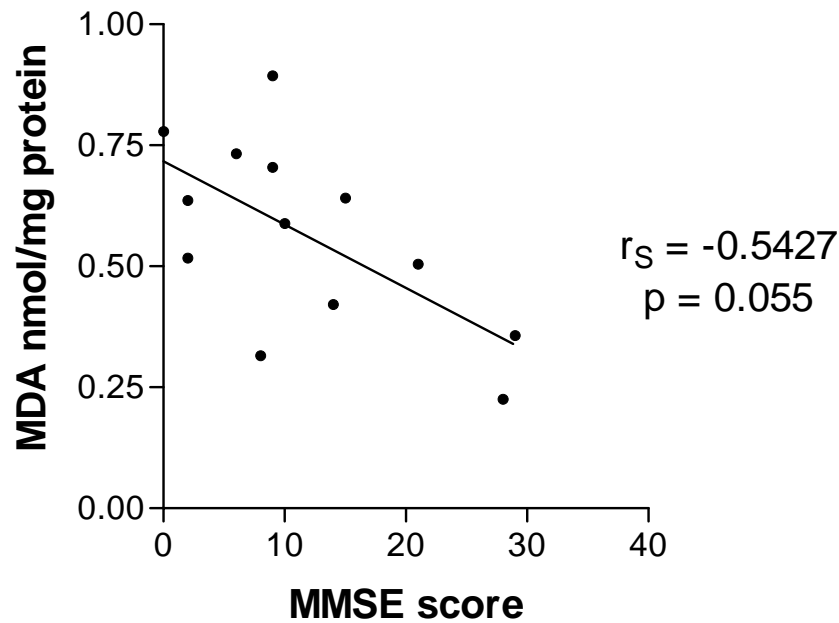


Figure 3.50: Correlation between MDA levels and MMSE score.

MDA levels (nmol/mg protein) were plotted against mini mental status examination (MMSE) score. Correlation: $r_s = -0.5427$, $p = 0.055$.

Levels of HNE were however not linearly correlated with MMSE scores (Figure 3.51 a). Patients with low MMSE scores had rather lower levels of HNE. Therefore, the data was further evaluated according to MMSE score. The mean MMSE score in AD patients was 8.6, therefore groups of AD patients with low MMSE scores of 8 or less and with moderate MMSE scores of 9 or higher were evaluated (Figure 3.51 b). Levels of HNE in the moderate MMSE AD patient group were significantly higher compared to the control group and were also significantly higher compared to AD patients with low MMSE scores. Therefore, it can be speculated that HNE levels are higher in earlier stages of the disease and decrease with the progression of the disease.

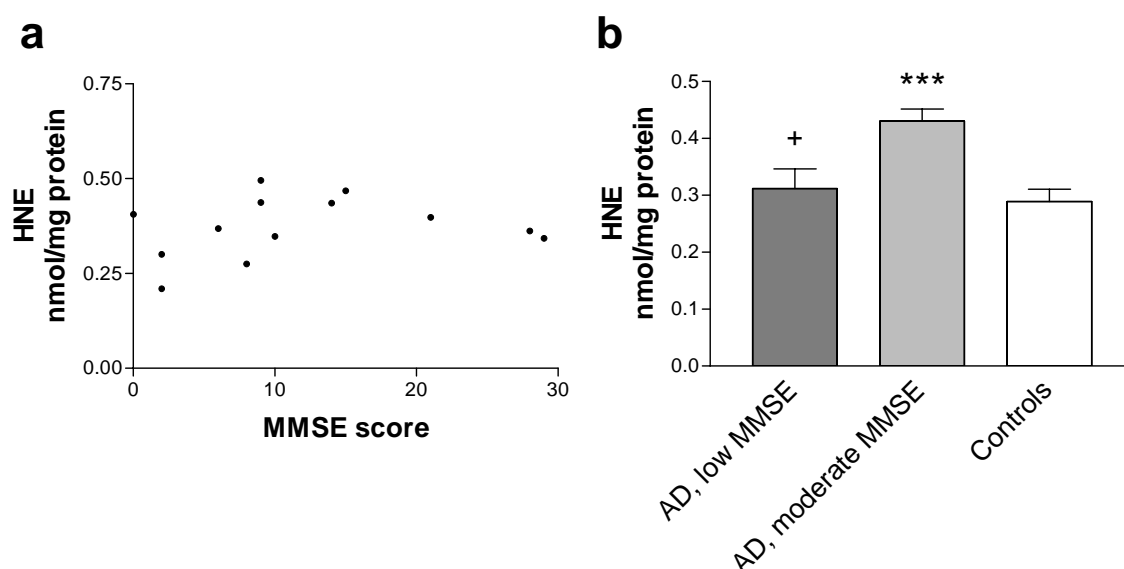


Figure 3.51: Correlation between HNE levels and MMSE score.

a. HNE levels (nmol/mg protein) were plotted against mini mental status examination (MMSE) score. No significant linear correlation was found between HNE levels and MMSE score.

b. Mean HNE levels (nmol/mg protein) vary with cognitive impairment. Samples were grouped according to cognitive impairment: AD patients were divided into two groups depending on MMSE score, either below the mean MMSE score of 8.6 (AD, low MMSE; dark grey bar; $n = 5$) or above 8.6 (AD, moderate MMSE; bright grey bar; $n = 6$). The mean of the control group is represented by the white bar ($n = 14$). One-way ANOVA: $p < 0.01$. Post hoc t-test + $p < 0.05$ low vs moderate MMSE AD patients, *** $p < 0.001$ moderate MMSE AD patients vs controls.

At the level of enzymatic antioxidant defense, no significant linear correlation with MMSE scores was observed for any of the enzymes Cu/Zn-SOD, GPx or GR, respectively (data not shown). When AD patients were divided into two groups with low and moderate MMSE scores, activities of Cu/Zn-SOD and GPx were highest in the moderate MMSE score group of AD patients, which showed a tendency towards significance for Cu/Zn-SOD (Figure 3.52 a) and was significant for GPx activity (Figure 3.52 b) compared to controls. In contrast to Cu/Zn-SOD and GPx activity, GR activity was highest in the group with low MMSE score, which was however not statistically significant (Figure 3.52 c).

Levels of MDA formation after *in vitro* stimulation showed no striking differences between the control group and each the low and moderate MMSE AD patient groups (data not shown).

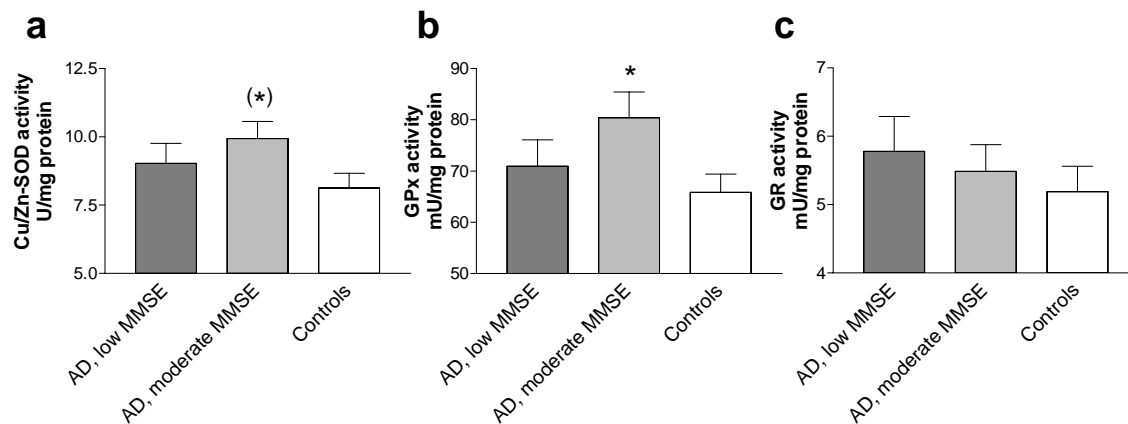


Figure 3.52: Activities of antioxidant enzymes in AD patients according to cognitive impairment.

Samples were grouped according to cognitive impairment: AD patients were divided into two groups depending on MMSE score, either below the mean MMSE score of 8.6 (AD, low MMSE; dark grey bar, $n = 5$) or above 8.6 (AD, moderate MMSE; bright grey bar; $n = 6$). The mean of the control group is represented by the white bar ($n = 14$).

a. Activity of Cu/Zn-SOD (U/mg protein). One-way ANOVA *n.s.* Post hoc *t*-test (*) $p < 0.1$ AD moderate MMSE vs controls.

b. Activity of GPx (mU/mg protein). One-way ANOVA $p < 0.1$. Post hoc *t*-test * $p < 0.05$ AD moderate MMSE vs controls.

c. Activity of GR (mU/mg protein). One-way ANOVA *n.s.*

It has to be noted that the MMSE score is dynamically changing during the disease progression with an average reduction of about 3 points per year (Salmon et al., 1990; Han et al., 2000; Ballard et al., 2001). Since the available MMSE score had in some cases been acquired several months prior to death, the time-span between the last MMSE assessment and death of the patient may have led to an additional loss in MMSE scores. Hence, the above effects were reevaluated with mathematically corrected MMSE scores assuming an average disease progression with a loss of 3 points in the MMSE per year. This evaluation produced essentially the same results (data not shown).

In summary, dynamic alterations in oxidative stress parameters occur during the progression of the disease – raising the possibility that the search for antioxidant pharmacological interventions aimed at a modification of antioxidant capacity may result in future AD therapies which can halt or at least slow the disease progression.

4 DISCUSSION

The results obtained in the course of this thesis allow for an estimation of (i) age-associated changes in oxidative stress parameters in mice and (ii) AD-relevant alterations in oxidative stress parameters studied in transgenic animals and human brain tissue. Aging is the most prominent risk factor for AD – with impact on the onset of disease in both the sporadic and the familial forms of AD – and aging also influences the accumulation of AD-associated neuropathology in transgenic mouse models. Hence the alterations in oxidative stress parameters observed in mice during aging and their relevance to the situation in humans will be discussed prior to the results on AD-associated changes.

4.1 Effects of aging and gender on ROS metabolism in C57BL/6J mice

4.1.1 Effect of aging on oxidative stress parameters in brain tissue

Among several organs, the brain is considered to be especially vulnerable towards oxidative stress due to its high metabolic rate, its high content of unsaturated fatty acids which are especially prone to free radical attack, the relative paucity of antioxidant defenses and limited ability to regenerate dead neuronal cells (Halliwell, 1992). As aged animals – rodents as well as non-human primates – exhibit cognitive deficits (Rapp and Amaral, 1989; Barnes et al., 1997) and as a loss of memory and cognitive abilities is present in aged humans (Ciocon and Potter, 1988; Albert, 2002), the contribution of oxidative stress to these age-associated deficits is a feasible hypothesis.

Paradoxically reduced levels of lipid peroxidation products in aged mice

Our results obtained on brain tissue homogenates from female mice during aging do however not unambiguously support the oxidative stress hypothesis. The hypothesis would predict that aging leads to increased accumulation of ROS. As high levels of ROS provoke oxidative damage to cellular components, markers for oxidative stress like lipid peroxidation products should be increased in aged animals. Yet in our study, lipid peroxidation products MDA and HNE as markers of oxidative damage to fatty acids are paradoxically lower in brains of aged animals (see Figure 3.1, page 83). Comparing these results with published data analyzing mouse brains with similar methods, it is obvious that several groups obtained conflicting results (Table 4.1).

Similarly conflicting results regarding changes in oxidative stress parameters with aging have been reported in other rodents, mostly rats. Thus, oxidative stress parameters show variable changes with age in different mammalian species and strains. Furthermore, striking differences seem to exist between different brain regions (Ramassamy et al., 2001). The conflicting results can be further due to animal maintenance conditions, different experimental procedures and tissue preparation techniques.

Table 4.1: Overview of studies on lipid peroxidation products during aging in mice.

change with aging	age groups analyzed	mouse strain, gender	Reference
increased	100-360 days	C57BL/6 X CBA/6J	(Cristiano et al., 1995)
increased	2, 10, 16, 20 months	NMRI, female	(Leutner et al., 2001)
increased	8, 11 months	C57BL/6N, female	(Liu et al., 2003)
increased	1.5-2, 19 months	Swiss albino, male	(Manda and Bhatia, 2003)
peak at 10 months	1, 4, 10, 18 months	CBA, female	(Sobocanec et al., 2003)
unchanged	2, 12, 28 months	C57BL/6 X DBA/2 F1 hybrids	(Ceballos-Picot et al., 1992)
unchanged	0-700 days	C57BL/6J X CB1 F1 hybrids	(de Haan et al., 1992)
unchanged	3, 25 months	C57BL/6 X DBA/2 F1 hybrids	(Bonnes-Taourel et al., 1993)
unchanged	3, 13 months	C57BL/6J, male	(Ramassamy et al., 2001)
unchanged	3, 12 months	C57BL/6J	(Oxenkrug and Requintina, 2003)

In the previous study by Leutner et al. on NMRI mice in our group (2001), essentially the same method of preparation and assay conditions have been employed. It is therefore possible that differences between C57BL/6J mice and NMRI mice in MDA levels during aging are due to inherent strain differences. Other studies have reported strain differences in oxidative stress parameters in mice (Ahotupa et al., 1993; Gerhard et al., 2002), albeit these studies did not analyze brain tissue and NMRI and C57BL/6J mice have not been compared directly. Nevertheless, strain differences likely account for some of the differences between our results and the study of Leutner et al. (2001).

Changes in activities of antioxidant enzymes

As oxidative damage can result from increased ROS production and/or insufficient detoxification, a higher antioxidant capacity of brain tissue from aged mice can explain the lower amounts of MDA and HNE. Our results on activities of antioxidant enzymes revealed that aging leads to increased antioxidant enzyme activities in murine brains. The three enzymes studied – Cu/Zn-SOD, GPx and GR – are affected differently by

DISCUSSION

aging: while GPx activity is steadily increasing up to an age of 25 months, Cu/Zn-SOD and GR activities rise until 19-22 months but decline in 25 months old animals to nearly the levels of 3 months old mice (see Figure 3.2, page 85). In general, increased or unchanged activities of several antioxidant enzymes have been reported by the majority of comparable studies in murine brains (Table 4.2). Cu/Zn-SOD activity was mainly reported to be increased, whereas glutathione-related enzymes GPx and GR displayed differential changes with aging.

Table 4.2: Overview of studies on antioxidant enzyme activities during aging in mice.

change with aging	age groups analyzed	mouse strain, gender	Reference
total SOD activity			
increased	3, 23-24 months	CD1, male	(Danh et al., 1983)
increased	1, 6, 12, 24 months	C57BL/6N, male	(Hussain et al., 1995)
decreased	12, 24 months	C57B1, male	(Mo et al., 1995)
Cu/Zn-SOD activity			
increased	0-700 days	C57BL/6J X CB1 F1 hybrids	(de Haan et al., 1992)
increased	3, 12, 28 months	C57BL/6 X DBA/2 F1 hybrids	(Ceballos-Picot et al., 1992)
increased	100-360 days	C57BL/6 X CBA/6J	(Cristiano et al., 1995)
increased	2, 10, 16, 20 months	NMRI, female	(Leutner et al., 2001)
unchanged	3, 23-24 months	CD1, male	(Danh et al., 1983)
unchanged	3, 18, 34 months	C57B1	(Cardozo-Pelaez et al., 1999)
GPx activity			
increased	1, 6, 12, 24 months	C57BL/6N, male	(Hussain et al., 1995)
increased	3, 12, 28 months	C57BL/6 X DBA/2 F1 hybrids	(Ceballos-Picot et al., 1992)
unchanged	0-700 days	C57BL/6J X CB1 F1 hybrids	(de Haan et al., 1992)
unchanged	3, 18, 34 months	C57B1	(Cardozo-Pelaez et al., 1999)
unchanged	2, 10, 16, 20 months	NMRI, female	(Leutner et al., 2001)
GR activity			
increased	2, 10, 16, 20 months	NMRI, female	(Leutner et al., 2001)
unchanged	3, 12, 28 months	C57BL/6 X DBA/2 F1 hybrids	(Ceballos-Picot et al., 1992)
decreased	12, 24 months	C57B1, male	(Mo et al., 1995)

As antioxidant enzyme activities vary between brain regions (Carrillo et al., 1992; Hussain et al., 1995), show a different cellular localization in neurons and glial cells (Delacourte et al., 1988; Ceballos et al., 1991; Wilson, 1997) and a discrete subcellular localization (Vitorica et al., 1984), the method of brain tissue preparation can probably explain some of the divergent findings. However, as the methods used in this thesis are the same as in the study by Leutner et al. on NMRI mice in our group (2001), subtle

differences between mouse strains seem to exist also at the level of antioxidant enzyme activities. While NMRI mice showed a large increase in Cu/Zn-SOD activity of 87 % already at an age of 10-12 months compared to 2-3 months old mice, the changes we observed in C57BL/6J mice were moderate with a maximum increase of 11 % in 19-22 months old animals compared to 3 months old mice. Furthermore, NMRI mice showed no significant elevation of GPx activity during aging, whereas GPx activity steadily increases in C57BL/6J mice with aging. Age-associated changes in GR activity are however similar in both mouse models, with a 22 % and 20 % increase in 19-22 months old NMRI or C57BL/6J mice, respectively, over the levels of young mice. Thus, NMRI mice differ from C57BL/6J mice in terms of greatly increased Cu/Zn-SOD activity but unchanged GPx activity with aging. Interestingly, the ratio of SOD versus GPx has been implicated in oxidative damage (de Haan et al., 1995), as increased SOD activity without concomitantly upregulated GPx activity would lead to accumulation of hydrogen peroxide. Therefore, increased MDA levels with aging in NMRI mice can be explained by an imbalance of Cu/Zn-SOD over GPx activity. Conversely, the simultaneously rising Cu/Zn-SOD and GPx activity in our study of C57BL/6J mice could be responsible for the decreased levels of lipid peroxidation products MDA and HNE during aging in this mouse strain.

From these results it can be postulated that the whole cascade of antioxidant enzymes is important in protection against oxidative damage. This is essentially not surprising, since the antioxidant network is a complex interplay between various factors, and in most studies a combination of several singular factors has shown the greatest benefit. In *Drosophila melanogaster*, the simultaneous overexpression of Cu/Zn-SOD and catalase led to increased life span accompanied by decreased oxidative protein damage (Orr and Sohal, 1994), whereas overexpression of Cu/Zn-SOD alone was ineffective (Orr and Sohal, 1993). Moreover, overexpression of Cu/Zn-SOD alone in mice even resulted in increased basal levels of lipid peroxidation products (Ceballos-Picot et al., 1992). Activities of antioxidant enzymes are obviously interrelated, as overexpression of Cu/Zn-SOD in mice can effect a increase in other antioxidant enzymes Mn-SOD, catalase and GPx (Przedborski et al., 1992). Cell culture studies with Cu/Zn-SOD overexpression produced similar results, where murine fibroblast produced higher levels of GR activity after transfection with Cu/Zn-SOD (Kelner et al., 1995a; Lee et al., 2001). However, overexpression of GPx does not alter activities of Cu/Zn-SOD (Kelner et al., 1995b), suggesting that Cu/Zn-SOD activity can affect downstream antioxidant

enzymes GPx and GR, while *vice versa* GPx does not affect upstream Cu/Zn-SOD activity. This generally supports the hypothesis that changes in a singular antioxidant enzyme can affect other related enzymes, especially if these are located downstream in the antioxidant enzymatic cascade.

Response to oxidative stimuli in vitro

As the total antioxidant capacity of brain tissue can not be estimated merely from the activities of antioxidant enzymes, experiments of *in vitro* stimulation of lipid peroxidation were conducted on the brain homogenates. Exogenously added ferric iron ions Fe^{3+} can be reduced to ferrous iron Fe^{2+} by superoxide radicals (see 1.1.1, equation (II), page 4). Ferrous iron subsequently propagates hydroxyl radical formation from hydrogen peroxide (see 1.1.1, equation (I), page 4), which reacts to initiate lipid peroxidation chain reactions (see 1.1.3.3, page 10 et seqq.). The net reaction is the Haber-Weiss reaction, where iron catalyses the decomposition of superoxide radicals and hydrogen peroxide to yield hydroxyl radicals. The radical attack on unsaturated fatty acids leads to lipid hydroperoxide formation, which are finally degraded to lipid peroxidation products like MDA. Thus, the amount of MDA formation after *in vitro* stimulation with ferric iron is an indirect measure for the free radical scavenging abilities of brain homogenates.

In our study on C57BL/6J mice, stimulation of MDA formation showed a U-shaped course during aging (Figure 3.3, page 86): samples from middle-aged 13-15 months old and aged 19-22 months old mice showed lower MDA formation after stimulation compared to young 3-4 months old mice, which is in good accordance with the increased activities of antioxidant enzymes in these age groups compared to young mice. Stimulation of lipid peroxidation was however increased in highly aged 25 months old mice compared to middle-aged and aged mice. The decline in activities of Cu/Zn-SOD and/or GR in 25 months old mice could explain this observation. As 25 months old animals show low Cu/Zn-SOD activity, superoxide radicals can accumulate in the sample homogenate giving rise to the Haber-Weiss reaction. Consequently, more radicals can participate in lipid peroxidation reactions. The diminished activity of GR would lead to depletion of reduced glutathione in the homogenate. Since glutathione can scavenge free radicals directly and is also a cofactor for the lipid hydroperoxide-reducing enzyme PH-GPx (Imai and Nakagawa, 2003), lack of reduced glutathione additionally favours lipid peroxidation reactions. Hence the effects of *in vitro*

stimulation of lipid peroxidation seem to reflect the overall free radical scavenging abilities of the different antioxidant enzymes.

Interestingly, in the study of Leutner et al. (2001), similar results have been obtained, where NMRI mice at 10-11 and 16-17 months displayed lower MDA formation compared to 2-3 months old mice after *in vitro* stimulation with ferric iron. Thereafter, at an age of 20-21 months, MDA formation rises again to nearly the levels of 2-3 months old mice. Such a U-shaped change of MDA formation after *in vitro* stimulation during aging was also observed in our studies on C57BL/6J mice. Hence – despite strain differences in C57BL/6J and NMRI mice in basal levels of lipid peroxidation products and activities of antioxidant enzymes SOD and GPx during aging – brain homogenates from C57BL/6J and NMRI mice display a similar reaction towards *in vitro* stimulation during aging. Although the results obtained in the different studies and mouse strains by our group correlate well, conflicting results have been reported by other researchers (see Table 4.3). Again, strain differences, animal maintenance conditions, different experimental procedures and tissue preparation techniques might serve to explain some of these variations.

Table 4.3: Overview of studies on *in vitro* stimulation of lipid peroxidation during aging in mice.

change with aging	age groups analyzed	mouse strain, gender	Reference
linearly increased	0-700 days	C57BL/6J X CB1 F1 hybrids	(de Haan et al., 1992)
increased	12, 24 months	C57B1, male	(Mo et al., 1995)
U-shaped course, lowest at 10-11 months	2, 10, 16, 20 months	NMRI, female	(Leutner et al., 2001)
peak at 12 months	3, 12, 28 months	C57BL/6 X DBA/2 F1 hybrids	(Ceballos-Picot et al., 1992)
unchanged	3, 13 months	C57BL/6J, male	(Ramassamy et al., 2001)

Conflicting reports from other labs – possible explanations

Apart from artefacts arising due to differences in methodologies between several research groups, the many divergent findings in various reports suggest two possible explanations for contradictory observations:

- The first possibility is that different animal species, strains, maintenance and environmental conditions have an impact on the adaption of organisms towards ROS during aging in such a way that the observed parameters – lipid peroxidation products and antioxidant enzymes – are differently regulated,

serving to keep other parameters in balance.

In this respect, the GSH/GSSG ratio seems to be a quite robust marker. Various studies have reported a decreased GSH content or decreased GSH/GSSG ratio in aged mouse brains (Chen et al., 1989; de la Asuncion et al., 1996; Rebrin et al., 2003; Wang et al., 2003; Manda and Bhatia, 2003). Although other studies have found increased or unchanged levels of GSH in various regions of aged mouse brains, synaptosomes or mouse brain mitochondria (Martinez et al., 1994; Hussain et al., 1995; Martinez et al., 1995), the GSH/GSSG ratio was not calculated in these studies. Thus, the most consistent results on oxidative stress parameters during aging in mouse brains have been obtained with the GSH/GSSG ratio so far.

Our results on alterations in activities of antioxidant enzymes with aging could at least hypothetically be reconciled with a decreased GSH/GSSG ratio. As Cu/Zn-SOD activity increases with aging, higher levels of hydrogen peroxide H_2O_2 should be produced from the SOD reaction, which are then processed by the GPx reaction under consumption of reduced glutathione. As the GPx activity is steadily increasing up to 117.7 %, but GR activity is only 104.2 % in 25 months old mice relative to young mice, it is conceivable that high GPx activity without simultaneously increased GR activity may result in an imbalance between the consumption and restoration of reduced GSH. Hence – although other factors have an impact on enzyme activity and availability of substrates *in vivo* – the ratio of GSH/GSSG could at least hypothetically be decreased in aged C57BL6/J mice.

- The other possibility to explain the conflicting results on activities of antioxidant enzymes and lipid peroxidation products in aging mouse brains may reside in the relatively mild changes that are observed in most studies. Although differences in lipid peroxidation products were as much as a maximum 33 % decrease for HNE in 25 months old mice, the changes in antioxidant enzyme activities ranged only up to a 19.7 % increase for GR activity in 19-22 months old animals compared to young mice. This maximum increase of less than 20 % on average may be difficult to be reproduced in further studies. Thus, if the changes in antioxidant enzyme activities during aging are rather small, the variances in different experiments may produce a slight but significant decrease in one study, while in another study the same variance may lead to a statistically

significant increase as final outcome.

Yet the observations in our group do not support this reasoning: although changes in activities of antioxidant enzymes may be somewhat subtle, the results of Leutner et al. (2001) and the results presented in this thesis regarding activities of antioxidant enzymes during aging in mouse brains consistently show an elevation of enzyme activities during aging. Furthermore, these effects have been reproduced later in independent studies by other researchers in our group (I. Scherping, S. Hauptmann, S. Schmitt-Schillig, personal communications), suggesting that the increase in antioxidant enzyme activities in mouse brains from either NMRI or C57BL/6J mice is a robust and reproducible observation.

Is the extent of antioxidant enzyme upregulation during aging sufficient?

A very important question arising from changes of antioxidant enzymatic defense with aging is whether the extent of upregulation is sufficient for prevention of oxidative damage. It has to be considered that the animals included in our study were free of visible tumours and showed no splenomegaly, thus representing rather “healthy aging”, which may explain why the extent of alterations observed during aging are rather modest. The maximum increase in antioxidant enzyme activities observed in mouse brain was 10.8 % for Cu/Zn-SOD and 19.7 % for GR activity at an age of 19-22 months, and 17.7 % for GPx activity at an age of 25 months compared to 3-4 months old mice. Are these changes of 10 to 20 % sufficient to be of functional relevance in aging brain tissue?

Our results on basal and stimulated levels of lipid peroxidation products support the notion that antioxidant defense is functionally intact and increased in aged mice, but as we did not analyze other factors apart from the three antioxidant enzymes it can not be definitely decided whether it is really the combination of increased Cu/Zn-SOD, GPx and GR activities that causes this protection against oxidative damage or whether another factor not analyzed in our studies mediates the effects. Interestingly, the extent of alterations in lipid peroxidation parameters were more pronounced, with a maximum decrease of 20 % for MDA and 33 % for HNE in 25 months old animals. Hence it must be considered whether a rather moderate increase in antioxidant enzyme activities can cause a more pronounced decline in oxidative stress parameters. An important point to be considered is that the enzymatic activities are measured in an assay where enzyme

substrate is provided in excess to have a linear correlation between substrate consumption and amount of enzyme present. It is however questionable whether these rather high substrate concentrations are ever reached inside cells *in vivo*. Assuming saturable Michaelis-Menten enzyme kinetics for the antioxidant enzymes (Fee and Bull, 1986; Kim and Kang, 1997; Sasaki et al., 1998; Hashida et al., 2002), the velocity of substrate consumption increases with rising substrate concentration. Hence, the amount of substrate, i.e. ROS, consumed in cells *in vivo* can be actually more pronounced than the relative increase in enzyme activities measured in the *in vitro* assays.

Furthermore, it has to be considered that over-active antioxidant enzymes may even have deleterious effects. Thus, highly increased activity of Cu/Zn-SOD can result in overproduction of hydrogen peroxide with prooxidant effects (Ceballos-Picot et al., 1992), especially when increased SOD activity is not counteracted by a rise in GPx and/or catalase activity (de Haan et al., 1996). Also, imbalanced activities of GPx and GR in highly aged mice could result in altered homeostasis of reduced GSH affecting the redox state of thiol-containing proteins, which may result in putative deleterious effects on cellular functions (Tanaka et al., 2001; Pastore et al., 2003). For these reasons, the relatively subtle increases in antioxidant enzymes during aging probably reflect *physiological healthy aging* and can be of functional relevance for efficient ROS detoxification.

Is oxidative damage associated with cognitive deficits?

Aging is associated with a slow deterioration of cognitive performance in humans (Ciocon and Potter, 1988; Albert, 2002), and moreover mild cognitive impairment in elderly is a risk factor for the clinical development of later AD (Grundman et al., 2004). Similar observations of impaired cognitive function with aging have been made in C57BL/6 mice and other rodents (Dean et al., 1981; Gallagher and Nicolle, 1993), and furthermore administration of antioxidants has been shown to protect against memory loss in aged mice (Joseph et al., 1998; Joseph et al., 1999; Martinez et al., 2000; Liu et al., 2003). Studies on humans have produced similar results (Perrig et al., 1997; Paleologos et al., 1998; Schmidt et al., 1998; Grodstein et al., 2000; Morris et al., 2002b). These findings raise the question whether oxidative damage contributes to age-associated cognitive deficits. Although increased oxidative damage at the level of lipid peroxidation could not be demonstrated in whole brain homogenates in our experiments in mice, the results do not rule out that oxidative damage can occur at discrete cellular

or subcellularly localized structures. It might be conceivable that the overall amount of lipid peroxidation products is decreased in brains as a whole, but that some small proportion of structures essential to cognitive function is nevertheless oxidatively damaged. Immunohistochemical detection of oxidatively modified proteins in aged rodent, primate and human brain tissue yielded highest staining in neuronal structures (Goyal, 1982; Horie et al., 1997; Gilissen et al., 1999; Vohra et al., 2001), therefore neurons might be oxidatively damaged although the brain as a whole does not reflect this damage. Furthermore, levels of lipid peroxidation products do not preclude that proteins or DNA are oxidatively modified. Hence our results do not rule out the possibility that neuronal structures can be affected by oxidative damage.

In this regard, it is interesting to consider the different subcellular localization of antioxidant enzymes: while Cu/Zn-SOD has been detected mainly in neurons (Ceballos et al., 1991; Zhang et al., 1993; Bergeron et al., 1996), GPx and GR have been detected in astrocytes (Damier et al., 1993; Knollema et al., 1996). Localization of GPx and GR in astrocytes is accompanied by an increased content of glutathione in these cells as compared to neurons (Rice and Russo-Menna, 1998), which coincides with the proposed important role of astrocytes in brain glutathione metabolism (Desagher et al., 1996; Dringen et al., 1999). We found that activity of Cu/Zn-SOD increases with aging until 19-22 months, whereas activity of GPx shows a steady increase with aging. It is therefore conceivable that rising Cu/Zn-SOD activity in aged mice leads to accumulation of hydrogen peroxide selectively in neurons, whereas increasing GPx activity offers only limited protection since it is localized mainly to astrocytes. This would result in the observed decrease in levels of lipid peroxidation products in whole brain homogenates from aged mice, while increased oxidative damage to neurons can not be ruled out at the same time. Thus, rising Cu/Zn-SOD activity in aging mice could contribute to selective oxidative vulnerability of neurons that would result in cognitive deficits. The importance of superoxide decomposition for maintenance of cognitive function has been elegantly demonstrated in a study by Liu and coworkers, where age-related cognitive deficits in female C57BL/6 mice could be reversed by systemic administration of brain permeable superoxide dismutase/catalase mimetics (Liu et al., 2003). Hence, the pharmacological augmentation of antioxidant defenses – thus giving further support to the aging-induced upregulation of antioxidant enzymes – may prove beneficial in the prevention of age-associated cognitive deficits.

Summary of aging-induced effects on oxidative stress parameters in brain tissue

Taken together, our observations on basal and stimulated lipid peroxidation parameters and activities of antioxidant enzymes suggest that brain aging in C57BL/6J mice is accompanied by several adaptive changes, which serve to reduce oxidative damage. As basal lipid peroxidation is decreased and activities of antioxidant enzymes are concomitantly upregulated in brains from aged mice, the antioxidant system obviously works efficiently in the detoxification of ROS, even in highly aged animals at 25 months of age. Only after *in vitro* stimulation of lipid peroxidation could we observe an increased susceptibility towards oxidative damage in highly aged compared to middle-aged and aged animals. Nevertheless, this increased susceptibility was not significant when comparing highly aged with young mice. Therefore, brains from female C57BL/6J mice seem to be quite resistant towards oxidative damage with aging – at least under the conditions in our experiments.

Although lipid peroxidation parameters can be influenced by an altered membrane lipid composition (Wood et al., 1984; Imre et al., 2000; Eckert et al., 2001d) or a rise in aldehyde-detoxifying enzymes (Cao et al., 1983; Martinez-Lara et al., 2003) during aging, which may have additionally affected the outcome of our measurements, our results nevertheless argue for a great importance of antioxidant enzymes in the protection against oxidative damage during aging.

4.1.2 Effect of aging on ROS levels in peripheral cells

Our studies on oxidative stress parameters in aging murine brains were extended to a peripheral cell model. We chose splenic lymphocytes since they offer several advantages:

- there seems to exist a close interaction between cells of the immune system and the central nervous system (Wekerle et al., 1987; Ringheim and Conant, 2004), suggesting that these peripheral cells can mirror some events in brain tissue,
- several studies on neurodegenerative diseases and brain aging in humans and mouse models have successfully used lymphocytes to monitor changes in brain tissue, including some studies of our own group (Eckert et al., 1994; Eckert et al., 1998; Schindowski et al., 2000; Eckert et al., 2001b; Gibson and Huang,

2002; Schindowski et al., 2003),

- lymphocytes represent easily accessible peripheral cells, which can be conveniently and selectively analyzed by flow cytometry,

and

- they can be used as a model of living cells, where ROS can be detected directly by the use of oxidation-sensitive fluorescent probes.

These advantages make lymphocytes a valuable tool to study age-related effects on brain oxidative stress parameters in a corresponding peripheral cell model.

Subtle increases in mitochondrial ROS during aging

The oxidative stress hypothesis would predict that levels of ROS rise with aging. However, no such effects could be observed in our study. Although lymphocytes show a tendency towards elevated oxidation of the ROS-sensitive dye DHR in middle-aged 13-15 months old animals, this effect was not further exacerbated in aged 19-22 months old animals (Figure 3.4, page 88). In the subset of CD4-positive lymphocytes, aged animals even display lower DHR oxidation than middle-aged animals. Therefore, the overall data on DHR oxidation during aging in splenic lymphocytes suggest that either increased ROS formation is not a major feature of aging – at least in these cell types under our conditions – or that antioxidant systems operate efficiently even in aged mice preventing excessive accumulation of ROS.

Other oxidation-sensitive fluorescent dyes DCFH-DA and DHE displayed no alterations with aging. Furthermore, staining of lymphocytes with R123, the oxidized form of DHR, revealed no age-associated changes, suggesting that the results obtained with DHR are not confounded by altered mitochondrial retention of R123 or by interactions via P-glycoprotein. In conclusion, neither altered mitochondrial function nor increased superoxide production are features of splenic lymphocytes in aging mice.

An overlap exists in the sensitivity of DHR and DCFH-DA towards oxidation by different ROS (see Table 2.3, page 68), as both dyes have been reported to be oxidized by peroxynitrite and peroxides (Bass et al., 1983; Kooy et al., 1994; Kooy et al., 1997; Hempel et al., 1999; Walrand et al., 2003). However, DCFH-DA seems to be oxidized mainly by cytosolic peroxides, whereas DHR has been reported to detect mainly mitochondrial ROS (Royall and Ischiropoulos, 1993; Diaz et al., 2003). From our results it can be estimated that cytosolic ROS are not increased during aging in splenic

lymphocytes, whereas mitochondrial ROS are slightly but not significantly elevated in aged animals. Importantly, these increased levels of mitochondrial ROS are probably not related to profound mitochondrial damage, since superoxide radical production and mitochondrial membrane potential, as evidenced by staining with DHE and R123, were not altered.

Increased NO levels in lymphocytes from aged female mice

The oxidation-sensitive dyes DHR and DCFH-DA have both been described to be oxidized by peroxynitrite, which is formed from a reaction of nitric oxide with superoxide radical anions. Although levels of superoxide radical anions are not increased during aging as evidenced by DHE staining, we additionally controlled for this confounding effect by staining of splenic lymphocytes with DAF-2-DA, a nitric oxide-sensitive dye. DAF-2-DA staining of lymphocytes increases with aging, while DHR and DCFH-DA oxidation do not show simultaneously increased oxidation, suggesting that under our conditions DHR and DCFH-DA are not oxidized to a significant degree by peroxynitrite in splenic lymphocytes.

Importantly, the increased NO levels with aging were only found in female mice (see also 4.1.3, page 165 et seqq.). The increased NO levels with aging may be related to deficient immune functions in aged mice (Quaglini and Ronchetti, 2001), as NO has been reported to inhibit T cell functions (van der Veen et al., 1999; van der Veen et al., 2000; Kahl et al., 2004), probably by inducing apoptosis (Bustamante et al., 2000; Oates and Gilkeson, 2004). However, the exact cell type as well as the nitric oxide synthase NOS subtype contributing to the increased NO production remains to be elucidated, as these factors have not been studied during aging in mouse spleenocytes so far. Only data on virus-transformed B cells, B-cell-lymphoma cells and leukemic T-cells are available showing iNOS expression in B-cell-derived cells (Mannick et al., 1994; Koide et al., 2003) and iNOS and nNOS expression in T-cell-derived cells (Kamimura et al., 2003). Furthermore, iNOS expression was identified in hepatic lymphocytes after hepatic injury (Leifeld et al., 2002). But from these data, no stringent conclusion can be drawn as to the type of NOS which causes the increase in nitric oxide levels in splenic lymphocytes from female mice during aging.

A putative contribution of antioxidant enzyme activities to the observed changes in ROS levels during aging?

Increased production of superoxide and hydrogen peroxide from mitochondria during aging has been described (Sohal and Sohal, 1991; Sohal et al., 1995). Superoxide radicals are however usually quickly decomposed to hydrogen peroxide H_2O_2 by mitochondrial Mn-SOD and cytosolic Cu/Zn-SOD, which has been detected in the intermembrane space of mitochondria (Okado-Matsumoto and Fridovich, 2001; Mattiazzi et al., 2002). Thus, if these enzymes work efficiently in aged cells and the increased production of H_2O_2 is not met by adequate activity of GPx in mitochondria (Timcenko-Youssef et al., 1985), the levels of hydrogen peroxide in mitochondria would be expected to rise, which can be detected by DHR oxidation. As GPx is also localized in even higher levels in cytosol compared to mitochondria (Timcenko-Youssef et al., 1985; Utsunomiya et al., 1991) and the cytosol is additionally protected by the presence of catalase in peroxisomes (Yamamoto et al., 1988; Makino et al., 1994), H_2O_2 reaching the cytosol is then efficiently removed – consistent with unchanged DCFH-DA oxidation in middle-aged mice. A further increase in the activities of antioxidant enzymes, especially GPx, with aging could explain why increased DHR oxidation is no longer observed in aged mice. In two recent studies in aging rats, antioxidant enzymes and mitochondrial chain activities in lymphocytes isolated from blood were directly compared with brain tissue. Importantly, the changes observed in lymphocytes paralleled those seen in brain tissue (Sandhu and Kaur, 2002; Sandhu and Kaur, 2003). These results demonstrate that changes during the aging process can be detected similarly in brain tissue and peripheral cells. Thus, it is possible that increased activities of antioxidant enzymes in lymphocytes, similar to our results on brain tissue, prevent excessive accumulation of ROS during aging.

Comparison with other reports

Only few other comparable studies on aging lymphocytes and ROS have been published so far. In previous studies by Leutner in our group, young (2-3 months) and aged (20-21 months) female NMRI mice were employed. Similar to our findings, DHR oxidation was not altered by aging in splenic lymphocytes (Leutner, 2001). Lymphocytes isolated from blood showed however elevated DHR oxidation in aged mice. This suggests that splenic lymphocytes have different properties compared to circulating cells – which is probably related to a different state of maturation and lower exposure of splenic

lymphocytes to stimulants like cytokines compared with circulating lymphocytes.

Studies on rat lymphocytes have shown elevated R123 staining in splenic lymphocytes from aged animals (Pieri et al., 1993) indicating that cells from aged animals display higher mitochondrial number and mass. Furthermore, rat lymphocytes displayed higher levels of oxidative damage to lipids and proteins (Tian et al., 1995). Studies on peripheral lymphocytes in humans have reported decreased activities of mitochondrial chain complexes with aging (Drouet et al., 1999), increased DHR oxidation (Leutner, 2001) and increased oxidative damage (Barnett and King, 1995; Piperakis et al., 1998; Mendoza-Nunez et al., 2001). Thus, the results we observed in murine splenic lymphocytes during aging are not in line with the majority of other comparable studies – which may be either due to specific properties of splenic lymphocytes, as stated above, or due to a relative resistance of C57BL/6J mice to age-induced ROS accumulation and oxidative damage, which had already been observed in brain tissue from these mice (see 4.1.1).

Summary of the effects of aging on ROS levels, lipid peroxidation products and antioxidant enzyme activities in C57BL/6J mice

Overall, our results on peripheral cells and the observations in brain tissue from C57BL/6J mice suggest that aging leads to adaptive responses serving to keep ROS levels at a relatively constant level. Therefore, only a small but not significant increase in ROS levels was observed, which is probably met by an adequate upregulation of antioxidant enzyme activities during the aging process. This is supported by our results on levels of lipid peroxidation products, which were reduced in aged animals. As lipid peroxidative damage is a downstream event of ROS attack on cellular membranes, with antioxidant enzymes playing an important role in intercepting ROS, the lower levels of lipid peroxidation products in aged animals result from the increased antioxidant enzyme activities. In conclusion, our results underline a prominent role for the upregulation of antioxidant enzyme activities in the prevention of excessive ROS accumulation and oxidative damage during aging in C57BL/6J mice.

4.1.3 Gender differences in oxidative stress-related parameters in mice

Gender differences in brain tissue

Analysis of age-related effects on oxidative stress parameters in brain tissue and splenic lymphocytes from male and female C57BL/6J mice during aging revealed distinct gender differences in some parameters. In brain tissue, levels of lipid peroxidation products MDA and HNE are reduced in female compared to male mice (Figure 3.5, page 90), and conversely, female mice show higher activity of the antioxidant enzyme glutathione peroxidase across all age groups (Figure 3.6, page 90). This suggests that brains from female mice are less susceptible towards oxidative damage due to higher antioxidant GPx activity. This is further supported by the results of *in vitro* stimulation of lipid peroxidation, where MDA formation is lower in brain homogenates from female mice (Figure 3.7, page 91). Thus, the higher antioxidant enzyme activity of GPx seems to exert a protective effect against increased lipid peroxidation under basal as well as stimulated conditions in female mice.

GPx activity has been shown to be protective against lipid peroxidation in *in vitro* systems (McCray et al., 1976; Ursini and Bindoli, 1987). Higher GPx activity results in more efficient detoxification of hydrogen peroxide, which can form hydroxyl radicals in Fenton reactions. Thus by scavenging of hydrogen peroxide, oxidative damage to lipids due to Fenton chemistry can be prevented. However, the gender differences in GPx activity and lipid peroxidation products could also be mediated by the opposite effects, i.e. that increased lipid peroxidation levels in male mice lead to a reduction of GPx activity, as HNE has been reported to lead to an inhibition of GPx activity *in vitro* (Bosch-Morell et al., 1999). It must be noted that HNE can also inactivate GR activity (Vander Jagt et al., 1997), but GR activity was not impaired in male mice. This can only be reconciled with our observations if (i) either these *in vitro* interactions of HNE with GPx and GR are not relevant *in vivo* at all, or (ii) the concentrations required for inactivation of GR are much higher than for GPx or (iii) a distinct subcellular localization of the respective enzyme and HNE leads to selective inactivation of only GPx in male mice.

Apart from GPx activity, other factors may have an additional impact on reduced levels of lipid peroxidation products in female mice. For example, the activity of glutathione-

S-transferase, which can lead to elimination of lipid peroxidation aldehydes by conjugation with glutathione, has been reported to be elevated in brains from female mice (Das et al., 1981). Thus, hormonal and/or genetic factors have a profound impact on antioxidant defense and parameters of oxidative damage in mouse brains.

From the available literature on similar observations, the contribution of gonadal hormones is difficult to elucidate. Although estrogen has been described to increase expression of antioxidant enzymes MnSOD and GPx in several tissues in animal models and humans (Igarashi et al., 1983; Salminen et al., 1988; Massafra et al., 2000; Akcay et al., 2000; Barp et al., 2002; Sobocanec et al., 2003; Borrás et al., 2003), these findings are controversial (Bolzan et al., 1997; Rush and Sandiford, 2003). Similarly, despite strong evidence that estrogens can influence activities of GPx, our results can not be explained by an effect of female gonadal hormones, as a gender difference in GPx activity was still present in 19-22 months old mice, where most female mice should be acyclic and have reduced estrogen levels (Nelson et al., 1995; Frick et al., 2000). Another possibility would be that male gonadal hormones specifically lead to a reduction of the activity of GPx, but again conflicting results have been reported (Azevedo et al., 2001). In summary – although we did not measure levels of gonadal hormones in our studies – a direct influence of gonadal hormones modulating activity of GPx seems unlikely. Therefore, other unknown factors – apart from direct effects of gonadal hormones – may be related to the sustained gender differences in oxidative stress parameters during aging in our study.

Gender differences in ROS levels in splenic lymphocytes

In good accordance with the increased GPx activity and decreased levels of lipid peroxidation products in brain tissue from female mice compared to male mice, splenic lymphocytes similarly exhibited lower DHR oxidation in samples from female mice (Figure 3.8, page 92). Of note, only DHR oxidation, but not oxidation of other ROS-sensitive dyes DCFH-DA or DHE was different between the sexes. Also, staining of lymphocytes with R123 revealed no gender differences, suggesting that mitochondrial functions are not altered. The selectively reduced oxidation of DHR in splenic lymphocytes from female mice may be related to the higher GPx activity in females. If the increased activity of GPx observed in brain tissue from female mice is also present in lymphocytes, in accordance with other studies (Sandhu and Kaur, 2002), hydrogen peroxide generated from mitochondria would be efficiently detoxified by GPx activity

in mitochondria (Vitorica et al., 1984; Timcenko-Youssef et al., 1985) before reaching the cytosol. Thus, mitochondrial DHR oxidation would be decreased in female mice. The importance for GPx activity in protection against mitochondrial oxidative stress was also demonstrated in GPx knockout mice, which display increased mitochondrial peroxide release and oxidative damage (Esposito et al., 2000). The gender difference is no longer detectable in the cytosolic compartment as evidenced by unchanged DCFH-DA oxidation between males and females, since (i) cytosolic GPx activity is higher than mitochondrial GPx activity (Timcenko-Youssef et al., 1985) resulting in efficient ROS decomposition even in male mice and (ii) cytosolic hydrogen peroxide can additionally be inactivated by the catalase reaction.

A profound gender difference was furthermore noticed after DAF-2-DA staining, where lymphocytes from females display higher DAF-2-DA oxidation indicating higher levels of nitric oxide. This gender effect was not detectable in young animals but was pronounced in aged animals. Although estradiol treatment has been described to increase nitric oxide levels via transcriptional activation of nNOS and eNOS in several studies on cell culture (Garcia-Duran et al., 1999; Chiueh et al., 2003; Lee et al., 2003b) and after *in vivo* administration (Weiner et al., 1994), a direct influence of estrogen in our study is unlikely. Nitric oxide levels were significantly increased only in aged female mice at 19-22 months of age, which is much older than the average age at loss of cyclicity at 13-15 months of age in C57 mice (Nelson et al., 1995). Therefore, the vast majority of female mice at 19-22 months of age should be acyclic due to ovarian failure (Frick et al., 2000). Consequently, estrogen levels would be expected to be decreased in these mice, which makes the above-mentioned effect of estrogen on nitric oxide levels unlikely in our model. Vice versa, reduced NO levels by androgens (Singh et al., 2000) are insufficient to explain our findings, as gender differences in NO levels caused by androgens should also have been detected in young mice. Furthermore, the overall data on the NO-modulating effects of androgens is controversial (Virdis et al., 2002). While tissues related to cardiovascular function and neuronal cells have been widely studied with respect to a hormonal influence on nitric oxide levels, studies utilizing lymphocytes are scarce (Pehlivanoglu et al., 2001), and the effects of gonadotropins on lymphocyte function during aging have not been explored. For these reasons, the observation that nitric oxide levels are selectively increased in lymphocytes from aged female mice can not be explained by the available literature.

It would be of interest to investigate if similar changes in nitric oxide levels with aging

and gender differences can be found in brain tissue, as nitric oxide is an important modulator of neuronal activity (Bredt, 1999) and has been suggested to play a role in brain aging in rodent models as well as humans (Tohgi et al., 1999; Siles et al., 2002). Interestingly, improvement of cognitive function by estrogen replacement therapy in humans has been suggested to be mediated by increased nitric oxide production (Lopez-Jaramillo and Teran, 1999). Therefore, the effect of nitric oxide production on the aging brain and the modulation by gender provides an interesting field for further studies.

Summary of gender differences in oxidative stress-related parameters

In summary, gender differences in oxidative stress-related parameters can be detected in brain tissue – where female mice exhibit lower levels of lipid peroxidation products along with higher antioxidant activity of GPx – and in splenic lymphocytes – where DHR oxidation is similarly reduced in cells from female mice. Hence both brain tissue and splenic lymphocytes display similar gender differences – with female mice showing better protection against oxidative damage and ROS accumulation. Therefore, our results support the notion that central effects in brain tissue can also be mirrored in peripheral lymphocytes.

In addition, our results indicate that activity of the antioxidant enzyme GPx is causally related to the observed gender differences, i.e. that female mice are seemingly provided with a higher protection against ROS accumulation and oxidative damage compared to male mice. In good accordance with our previous observations on the impact of aging in C57BL/6J mice, where rising antioxidant enzyme activities proved to be protective against oxidative damage, increased antioxidant GPx activity can similarly cause lower lipid peroxidation in female mice. Therefore, our results underscore the important role of antioxidant enzyme activity in the protection against ROS accumulation and oxidative damage.

Furthermore, a gender-disparate effect on nitric oxide levels during aging was observed, as increased levels of nitric oxide in lymphocytes could be detected only in female mice during aging. Hence, apart from increased GPx activity, an antioxidant effect of nitric oxide could additionally contribute to decreased lipid peroxidation and/or mitochondrial ROS formation in aged female mice (see Figure 4.1).

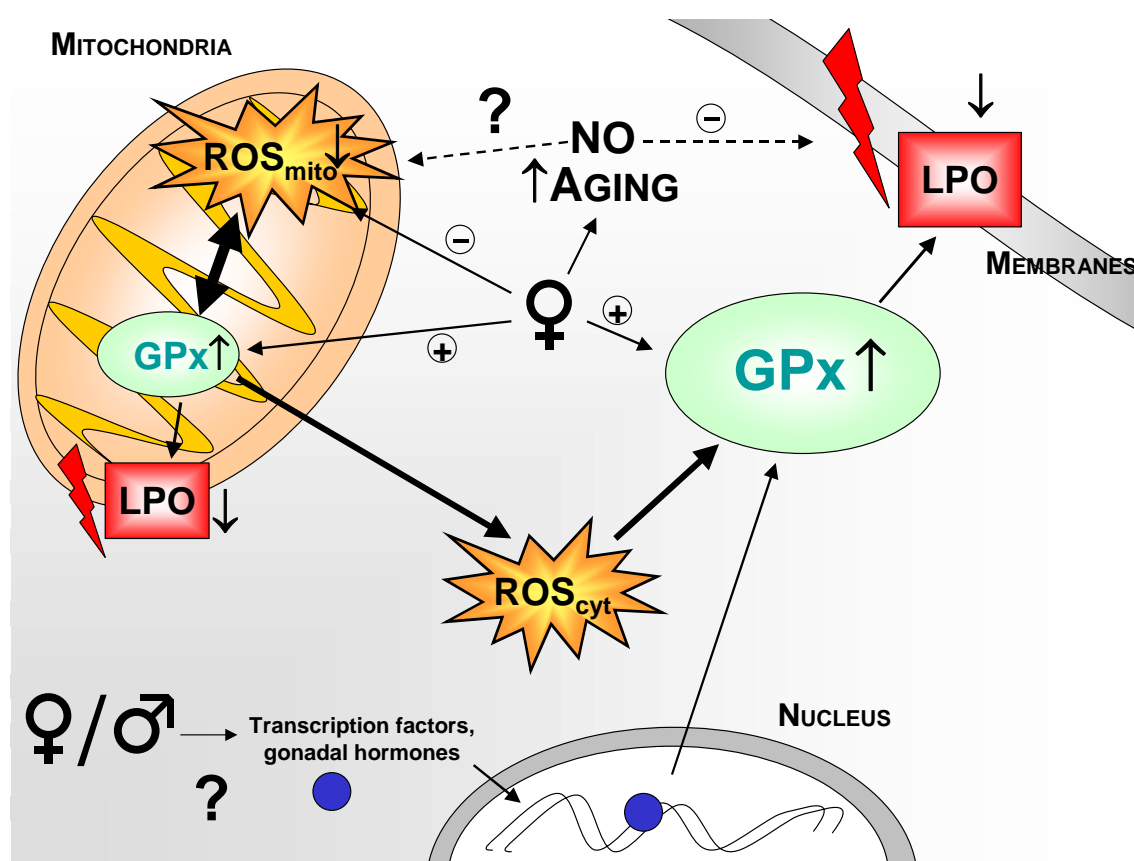


Figure 4.1: Summary of gender differences in oxidative stress-related parameters observed in mice.

Abbreviations: ROS_{mito}/ROS_{cyt} = mitochondrial/cytosolic reactive oxygen species; GPx = glutathione peroxidase; LPO = lipid peroxidation.

Female gender affects the activity of GPx, which results in efficient detoxification of ROS in mitochondria and in the cytosol. As a result, levels of lipid peroxidation products, which arise from downstream events of ROS attack on cellular membranes, are reduced in female mice. Exactly how increased GPx activity in female mice or vice versa reduced GPx activity in male mice is mediated – e.g. by an influence of gonadal hormones or gonadotropins on gene transcription – remains to be elucidated. Furthermore, rising nitric oxide levels during aging in female mice may contribute to decreased levels of lipid peroxidation products and/or mitochondrial ROS.

Gender differences between males and females in animal models but also in humans are becoming increasingly recognized in recent years. Differences in mitochondrial ROS production, antioxidant enzymes and/or oxidative damage between males and females have been reported in animals and humans (Das et al., 1981; Carrillo et al., 1992; Mendoza-Nunez et al., 2001; Proteggente et al., 2002; Sobocanec et al., 2003; Borrás et al., 2003), suggesting that female gender permits increased protection against oxidative damage in various species. Furthermore, these findings were linked with a simultaneously increased lifespan in females (Vina et al., 2003). The decreased mitochondrial ROS production in females has been suggested to be due to the activation of neuronal nitric oxide synthase expression by estrogen (Chiueh et al., 2003), followed by increased levels of antioxidant protein thioredoxin which in turn supposedly affects

expression of antioxidant genes Mn-SOD and GPx. Increased levels of nitric oxide by estrogens have been implicated in the relative protection of females against cardiovascular disease before menopause (Virdis et al., 2002). Interestingly, we also observed increased nitric oxide levels in splenic lymphocytes from female mice, however this difference was only found in aged mice, making a direct influence of estrogen unlikely, as stated above. As GPx has been localized to cytosol but also to mitochondria (Timcenko-Youssef et al., 1985), the increased enzyme activity may contribute to protection of mitochondria against damaging ROS and may thus be of relevance for a higher life span in females. It has to be noted, however, that in C57BL/6 no such higher life span in females has been reported so far (Storer, 1966; Goodrick, 1975). Therefore, it is difficult to assess the functional relevance of increased GPx activity in brains from female C57BL/6 mice with respect to a different life span of male and female mice. However in humans, women have an increased life expectancy compared to men (Smith, 1989). Thus, it would be of interest to study if similar gender differences in oxidative stress parameters during aging can be found in humans and to elucidate a possible functional role for a gender-disparate life span.

4.1.4 Summary of aging-induced effects on ROS and oxidative stress parameters in C57BL/6J mice

Although a vast body of evidence has been accumulated that mitochondrial alterations and oxidative stress are a major feature of aging, our results do not unequivocally support these findings.

Mitochondria are considered to be the most important intracellular source for ROS under physiological conditions (Boveris and Chance, 1973; Wei, 1998) and aging leads to increased mitochondrial proliferation (Barrientos et al., 1997; Lee et al., 2000; Pesce et al., 2001). This may be in an attempt to compensate for decreased activities of mitochondrial respiratory chain activities (Bowling et al., 1993; Ojaimi et al., 1999). Thus, an increased amount of mitochondria, which are however impaired in their function, might contribute to observations that ROS production from mitochondria increases during aging (Sohal and Sohal, 1991; Sohal et al., 1995).

Despite the vast body of evidence for increased ROS production during aging, we were not able to detect profoundly increased levels of ROS in splenic lymphocytes from mice – only a slight but not significant elevation of DHR oxidation was observed. Similarly

we did not find increased oxidative damage in brain tissue, in contrast, markers of lipid peroxidation products were even reduced in aged mice. Therefore, at least in C57BL/6J mice and under our conditions, profound oxidative damage does not seem to be a major feature of the aging process. However, our results may also be interpreted in a completely different view: as aging is a process that turns young animals into old ones, it can be argued that some factors must be present in young animals that determine their aging process (Dozmorov et al., 2002). Therefore, increased levels of lipid peroxidation products in young mice may be regarded as a *causative* factor for the aging process in these animals.

Furthermore, ROS must play some role during aging because changes could be observed at the levels of antioxidant enzymes in brain tissue. Activities of antioxidant enzymes Cu/Zn-SOD, GPx and GR in brain tissue are elevated during aging, and it is well established that ROS can induce antioxidant responses in cell culture models (Shull et al., 1991; Yoshioka et al., 1994; Tate, Jr. et al., 1995; Jornot and Junod, 1997; Rohrdanz et al., 2001). Hence, upregulation of antioxidant enzyme activities *in vivo* may be mediated by only a minimal accumulation of ROS during aging, as a slight but not significantly increased DHR oxidation was observed in splenic lymphocytes. Overall, it seems that compensatory changes in antioxidant defense can counteract excessive ROS accumulation and lipid peroxidation damage in aging tissues.

This is supported by the gender differences observed in brain tissue and splenic lymphocytes, where female mice exhibit higher GPx activity accompanied by reduced levels of lipid peroxidation products in brain tissue and reduced DHR oxidation in splenic lymphocytes compared to male mice. These gender differences demonstrate the importance of antioxidant enzymes in protection against lipid peroxidation and ROS accumulation.

Conclusion

In summary, activities of antioxidant enzymes play an essential role in ROS detoxification and seem to be modulated in such a way as to keep ROS levels at a relatively constant steady-state level during aging. As lipid peroxidation is a downstream event of ROS accumulation and antioxidant enzymes modulate the reactions leading from ROS to lipid peroxidation products, higher activities of antioxidant enzymes result in decreased levels of lipid peroxidation products in aged animals (summarized in Figure 4.2).

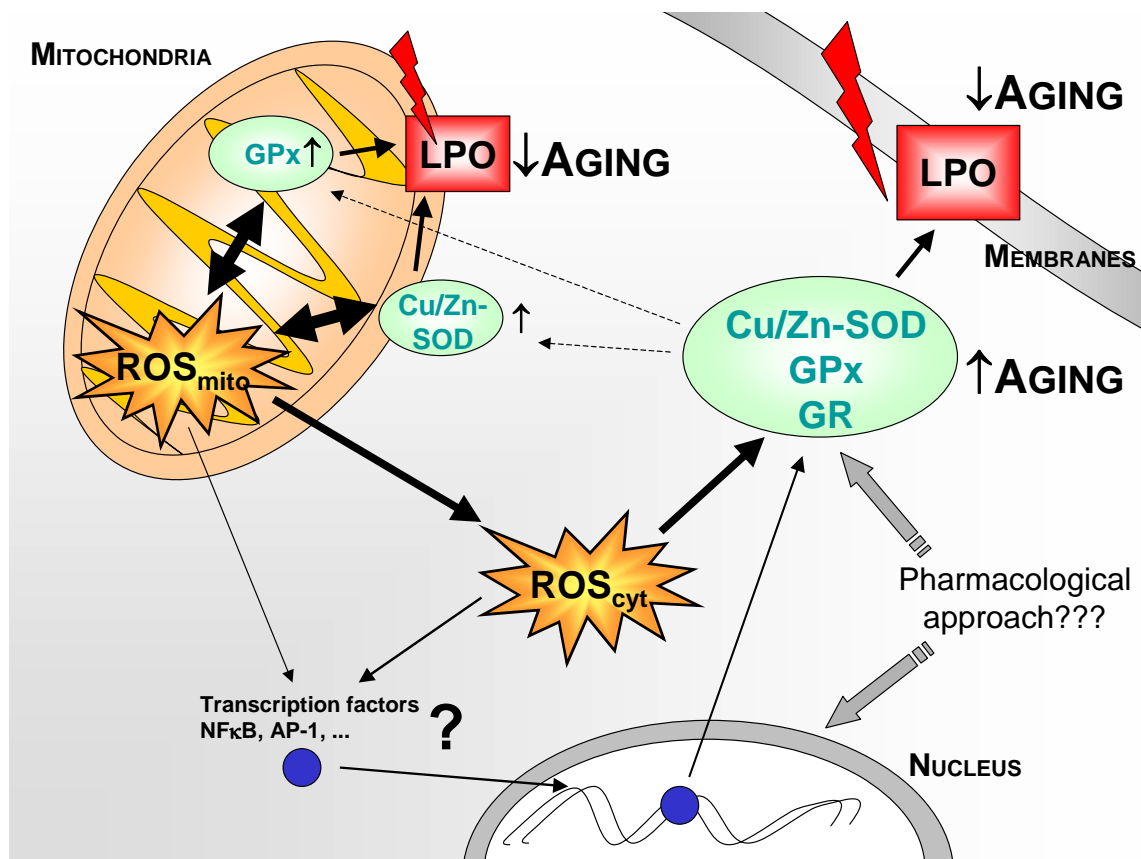


Figure 4.2: Summary of changes in oxidative stress-related parameters during aging observed in mice.

Abbreviations: ROS_{mito}/ROS_{cyt} = mitochondrial/cytosolic reactive oxygen species; Cu/Zn-SOD = superoxide dismutase, GPx = glutathione peroxidase, GR = glutathione reductase; LPO = lipid peroxidation.

During aging in mice, antioxidant enzymes are upregulated. This results in efficient detoxification of ROS in mitochondria (by GPx in the matrix and Cu/Zn-SOD in the intermembrane space) and in the cytosol (Cu/Zn-SOD, GPx and GR). As a result, levels of lipid peroxidation products, which arise from downstream events of ROS attack on cellular membranes, are reduced in aged mice. How antioxidant enzyme upregulation takes place – e.g. by activation of ROS-sensitive transcription factors – remains to be elucidated. Exogenous augmentation of antioxidant enzyme activities may represent a pharmacological approach to ameliorate age-induced deficits.

As our study on brain tissue does not allow for an estimation of effects in single cell types in brain tissue or the subcellular localization, it remains to be resolved whether oxidative damage can be present in discrete structures, e.g. selectively in neurons or subcellularly located to mitochondria, which are not separately detected by our methods utilizing whole brain homogenates.

Since activities of antioxidant enzymes increase during aging, it would be interesting to investigate whether a further augmentation of antioxidant defenses is of benefit in preventing age-associated deficits. It is noteworthy that, in a comparison of several mammalian species, activity of SOD was positively (Ono and Okada, 1984) and the peroxide-generating potential of tissues was negatively correlated with life span (Cutler,

1985). Therefore, augmentation of antioxidant defenses might prolong life span. In recent years, caloric restriction has been proposed as a means of prolonging life span in rodents (Sohal and Weindruch, 1996) and non-human primates (Lane et al., 2001). Caloric restriction reduces oxidative damage in rodents (Youngman et al., 1992; Tian et al., 1995) and increases activities of antioxidant enzymes (Cadenas et al., 1994; Sreekumar et al., 2002). In brain tissue, exogenous administration of SOD mimetics reduced oxidative stress and improved cognition in aged mice (Liu et al., 2003). Pharmacologically, augmentation of endogenous enzymatic antioxidant defense in brain tissue was for example reported by nerve growth factor NGF in aged rat brains (Nistico et al., 1992) or by monoaminoxidase B inhibitors deprenyl and rasagiline, where upregulation of antioxidant enzymes was also associated with a concomitant prolongation of life span (Kitani et al., 1994; Carrillo et al., 2000). Furthermore, transcriptional activation or upregulation of antioxidant enzyme activities could be shown with *Ginkgo biloba* extract *in vivo* (Aricioglu et al., 2001; Bilgihan et al., 1994; Bridi et al., 2001; Lin and Chang, 1997; Seif-el-Nasr and El-Fattah, 1995) and *in vitro* (Soulie et al., 2002) or with *Ginseng* components (Kim et al., 1996). These results suggest that upregulation of antioxidant defense can hypothetically contribute to the neuroprotective properties of extracts from these medicinal plants (Youdim and Joseph, 2001; Müller and Chatterjee, 2003; Eckert et al., 2003a).

Although only experimental evidence is available so far, the available literature and our results suggest that pharmacological augmentation of antioxidant enzyme activities may therefore prove beneficial in age-associated neurodegenerative diseases. However, more studies correlating antioxidant effects with cognitive performance *in vivo* are needed. And it has to be kept in mind that – although age-associated deficits may be prevented by such strategies – not merely an extension of life span is warranted, but rather an extension of *healthy* life span and augmentation of quality of life during aging should be the principal aims of studies in humans.

4.2 Oxidative stress-related parameters in transgenic mice bearing FAD mutations

In the course of this thesis, different transgenic mouse models based on familial AD mutations in either PS1 or APP could be studied. Although familial AD mutations are only found in few families worldwide, carriers of these mutations display an early onset of dementia with clinical and neuropathological symptoms indistinguishable from the much more common sporadic form of AD. As familial AD mutations consistently lead to an increased production of amyloid beta A β in humans, the amyloid cascade hypothesis has been proposed as the underlying mechanism for the development of sporadic as well as familial AD (Hardy and Higgins, 1992; Hardy and Selkoe, 2002). Consequently, transgenic mice with increased A β formation represent one of the best models to study putative toxic mechanisms of A β accumulation *in vivo*.

Different types of mice were used in this thesis, displaying different formation and accumulation of A β . Highest human A β levels are observed in Thy1-APP and in APP23 transgenic mice, while PDGF-APP/PS1 transgenic mice display intermediate A β accumulation and single PDGF-APP transgenic mice show only very low formation of human A β and no accumulation during aging. In contrast to the different APP transgenic mice, PS1 transgenic mice only possess endogenous rodent APP and can consequently only form mouse but not human A β . Thus, the different mouse models allow for an elucidation of the effects of PS1 mutations and rodent A β on oxidative stress parameters separately from the effects of various degrees of human A β accumulation in the different APP transgenic mice.

4.2.1 Effect of PS1 mutations on oxidative stress-parameters in transgenic mice

The mutant PS1 transgenic mice studied in this thesis show no formation of plaques derived from endogenous rodent A β during aging, which is probably due to different aggregation properties of human and rodent A β (Dyrks et al., 1993) or due to further unknown factors in brain microenvironment preventing plaque accumulation under normal conditions in rodents (Wyss-Coray et al., 1997; Little et al., 2004). Despite the lack of human A β peptide, which is considered the toxic species in the amyloid hypothesis, PS1 mutant mice have been described to display neurodegeneration (Chui et

al., 1999; Sadowski et al., 2004) and cognitive deficits (Pak et al., 2003), suggesting that either presenilin mutations have deleterious effects unrelated to A β production or that the endogenous rodent A β displays toxicity, which would be consistent with neurodegeneration observed in mice overexpressing rodent A β (LaFerla et al., 1995).

In order to elucidate the specific effect of a familial PS1 mutation compared to the effect of human wildtype PS1 overexpression in mice, two types of transgenic mice were used in our studies: PS1wt transgenic mice expressing human wildtype PS1 and PS1M146L transgenic mice expressing human mutant PS1. PS1wt transgenic mice are even better controls than non-transgenic mice for effects observed in PS1M146L mice, as the expression of wildtype PS1 alone has been reported to exhibit cytotoxic effects in some cell culture models (Wolozin et al., 1998; Hashimoto et al., 2002). Both transgenes are expressed under the HMG-CoA reductase promotor, which leads to a strong expression in neurons but also ubiquitous expression in other tissues (Leutner et al., 2000). Hence peripheral cells like lymphocytes could be studied for direct analysis of transgene effects on ROS levels in live cells *ex vivo*.

Analysis of brain tissue

In brain tissue from PS1wt or PS1M146L transgenic mice, no striking effects on the levels of lipid peroxidation products as indicator of oxidative damage could be observed compared to non-transgenic mice in any age group. Merely in the group of aged 19-22 months old mice, HNE levels were increased in PS1M146L mice compared to PS1wt mice (Figure 3.9, page 96). Thus it seems that PS1wt might exert a protective effect against lipid peroxidation at the level of HNE. However, due to rather large variation of values, differences were only significant in a direct comparison of PS1wt with PS1M146L transgenic mice, but not statistically significant in ANOVA. As no further animals of this high age were available during the time of this thesis, the results on HNE levels should be considered preliminary before they can be independently reproduced in another maybe larger cohort of animals.

At the level of antioxidant enzyme activities in brain tissue, no differences could be observed in any age group for any of the enzymes analyzed (Figure 3.10, page 97). This suggests that either a failure of other antioxidant defenses or more likely a mechanism of increased ROS production is related to the observed elevated HNE levels in brains from PS1M146L mice. This is further supported by the results of *in vitro* stimulation of lipid peroxidation, which was not different between the PS1 transgenic animals. Hence,

PS1 mutations do not cause deleterious effects via impairment of antioxidant defense mechanisms.

ROS levels in splenic lymphocytes

In order to characterize the respective type or subcellular localization of ROS that could be responsible for oxidative damage in brains from PS1M146L mice, levels of various ROS were further analyzed in splenic lymphocytes from these animals. Expression of the PS1 transgene could be detected by RT-PCR in these peripheral cells, confirming previous observations with Western Blot analysis of protein expression (Schindowski, 2001).

Aging sensitizes to the effect of the PS1 mutation

An effect of aging could be observed: while no differences in ROS levels measured by DHR oxidation were observed in cells derived from young animals neither under basal nor under stimulated conditions, lymphocytes from middle-aged PS1M146L animals showed unaltered basal ROS levels but increased ROS levels after serum deprivation (Figure 3.12, page 100), which represents a mild stress condition leading to apoptosis induction in lymphocytes (Kawanishi, 1997; Maucher et al., 1998). Finally, in cells from aged animals, even basal levels of ROS were increased (Figure 3.13, page 100). Thus, the aging process seems to sensitize towards the ROS-elevating effect of the PS1M146L mutation, which is evident in middle-aged animals only after mild stress but can be readily detected in aged mice even under basal conditions. Of note, the presence of PS1wt in transgenic mice did not alter ROS levels in lymphocytes, suggesting that the increased ROS levels are specifically caused by the presence of the FAD mutation. It can only be speculated which factors cause this age-associated higher susceptibility towards ROS accumulation in PS1M146L cells. A slight but not significant elevation of mitochondrial ROS levels was observed in murine splenic lymphocytes during aging (see 4.1.2, page 158). Nevertheless, this slight elevation of ROS levels may lower the threshold of oxidative homeostasis in aged cells, leading to an exaggerated ROS accumulation in PS1M146L-expressing lymphocytes from aged mice. This would furthermore be in accordance with our observations on brain tissue from C57BL/6J mice, where antioxidant enzymes are upregulated during aging, which occurs probably in response towards ROS formation in aged animals.

The PS1 mutation does not impair antioxidant defense

In good accordance with the unchanged activities of antioxidant enzymes in brains from these animals, no exacerbation of ROS formation could be detected in splenic lymphocytes after oxidative stimulation. Neither stimulation with hydrogen peroxide, which can lead to hydroxyl radical formation via Fenton reactions and mitochondrial damage (Dumont et al., 1999), nor stimulation with d-Ribose leading to glutathione depletion (Barbieri et al., 1994) resulted in profoundly elevated DHR oxidation as a measure of ROS accumulation in cells from PS1 transgenic mice (Figure 3.15, page 102, and Figure 3.17, page 105). Although ROS formation was increased after stimulation in lymphocytes, the relative increase in cells from PS1M146L mice was not substantially higher than the increase in cells from non-transgenic mice. Rather it seems that the increased basal DHR oxidation in PS1M146L mice is conserved after stimulation but not further exaggerated by the PS1 mutation. Expression of PS1wt led to a statistically not significant and only small increase in DHR oxidation after stimulation with a low concentration of hydrogen peroxide (1 μ M). As overexpression of wildtype PS1 in cell culture has been reported to predispose towards apoptotic stimuli (Wolozin et al., 1998; Hashimoto et al., 2002), it may be that the increased ROS formation after hydrogen peroxide stimulation is related to a similar hypersensitivity caused by PS1wt in our model. However, as the effect was not significant and no changes in antioxidant enzyme activities or *in vitro* stimulation of lipid peroxidation were observed in brains from PS1wt mice, this effects seems of minor importance.

Specific elevation of mitochondrial and cytosolic ROS but not superoxide radicals caused by the PS1 mutation

After an initial study of non-transgenic and PS1M146L transgenic mice, we had observed increased basal DHR oxidation in cells from aged PS1M146L mice. As various ROS are produced in living cells and can interact in complex reactions, we additionally conducted experiments in a second set of animals where lymphocytes were stained with several different ROS-sensitive dyes for an estimation of the exact type and the source of ROS that accounts for increased DHR oxidation caused by PS1M146L.

The increased oxidation of DHR is indicative of elevated mitochondrial ROS levels (Royall and Ischiropoulos, 1993; Ischiropoulos et al., 1999). Additionally, increased DHR oxidation is specific for the mutant PS1M146L, as oxidation of DHR was not altered in PS1wt transgenic mice bearing human wildtype presenilin (Figure 3.16 a,

page 104). Furthermore, cytosolic ROS levels measured by oxidation of DCFH-DA (Diaz et al., 2003) were similarly increased in PS1M146L mice, and DHR and DCFH-DA fluorescence showed a significant and positive linear correlation, indicating that both dyes are oxidized by the same ROS (Figure 3.16 c and d, page 104). DHR and DCFH-DA have been reported to be oxidized by hydrogen peroxide and peroxynitrite (Crow, 1997). In order to elucidate a possible contribution of increased peroxynitrite levels towards DHR and DCFH-DA oxidation, lymphocytes were additionally stained with DAF-2-DA and DHE, which are nitric oxide- and superoxide-sensitive dyes, respectively, detecting the precursors of peroxynitrite formation. DHE oxidation was not affected by the PS1M146L transgene, and DAF-2-DA showed only a slight but not significant trend towards increased oxidation (Figure 3.16 e and f, page 104), suggesting that peroxynitrite is not a major contributor to the increased DHR and DCFH-DA oxidation in lymphocytes from PS1M146L transgenic mice.

A possible confounding effect associated with DHR stimulation can arise from altered incorporation of the oxidized dye, R123, into mitochondria. This was controlled for by staining of lymphocytes directly with R123. R123 uptake into mitochondria was slightly but significantly increased in PS1M146L transgenic cells, which may contribute to the observed effects of increased DHR fluorescence (Figure 3.16 b, page 104). Nevertheless, the extent of increased R123 incorporation into mitochondria with 7.2 % relative to the mean values measured in cells from non-transgenic mice is not sufficient to explain the 42.1 % increase in DHR fluorescence. Therefore, the major part of increased DHR fluorescence must be due to oxidation of the dye.

The increased staining of PS1M146L-expressing lymphocytes with R123 could be due to stimulation of lymphocyte activation by elevated ROS levels. R123 has been described to be increasingly incorporated to mitochondria after stimulation of lymphocyte proliferation (Nairn et al., 1979; Ferlini et al., 1995). As ROS can mimic mitogenic signaling resulting in lymphocyte activation (Roth and Droge, 1987), the increased R123 incorporation into mitochondria may be secondary to activation of cells after ROS production caused by the PS1M146L mutation. Additionally, we detected a slight but not significant elevation of NO levels in PS1M146L-expressing cells, and NO can stimulate mitochondrial proliferation (Nisoli et al., 2003). Hence, increased NO levels can be reconciled with increased R123 staining due to mitochondrial proliferation in PS1M146L cells.

Similar vulnerability of lymphocyte subsets

As previous studies from our group and others had identified a different vulnerability of lymphocyte subsets to apoptosis during aging and in AD patients (Schindowski et al., 2002; Schindowski et al., 2003), we analyzed specific T-lymphocyte subsets from transgenic mice for a putative different formation of ROS. As a result, the effects of the PS1 mutation were not different in CD4- or CD8-positive lymphocyte subsets, suggesting that the transgene leads to similar effects independent of the cell type. However, we could observe that DHR oxidation was specifically higher in the CD4-positive lymphocyte subset compared to CD8-positive lymphocytes, independent of the transgene. Interestingly, CD4-positive splenic lymphocytes also display higher levels of basal apoptosis compared to CD8-positive cells (Schindowski, 2001), suggesting that the increased DHR oxidation observed in CD4-positive cells may be causally related to the apoptotic process in these cells.

Functional relevance of increased ROS levels in PS1M146L mice for apoptotic cell death

Apoptosis measurement in splenic lymphocytes from PS1 transgenic mice was conducted by Claudia Frey, utilizing the DNA dye 7-AAD as indicator of apoptotic cells (Schmid et al., 1994). Correlation of apoptosis levels in lymphocytes (percentage of apoptotic cells) with values of DHR oxidation in CD4-positive splenic lymphocytes from each individual animal resulted in a significant and positive linear correlation (Figure 3.14, page 101). Hence, in CD4-positive lymphocytes, where basal ROS levels are already higher than in CD8 cells, increased mitochondrial ROS may be functionally related to apoptosis. This is in good accordance with the emerging important role of mitochondria as the central executioners in apoptotic signaling (Kroemer et al., 1997; Susin et al., 1998). Mitochondrial damage can trigger apoptosis – probably via glutathione depletion (Macho et al., 1997) – followed by oxidation of mitochondrial thiols and loss of mitochondrial membrane potential (Marchetti et al., 1997), providing a functional link between increased mitochondrial DHR oxidation in CD4-positive lymphocytes and apoptosis. Of note, a similar higher sensitivity towards apoptosis in CD4 cells has been reported before in a study of peripheral lymphocytes from AD patients (Schindowski et al., 2003), suggesting that this cell type specifically reacts more sensitive towards ROS accumulation and cell death in AD-relevant models.

It is difficult to determine whether ROS initiate apoptosis or are a by-product of the

apoptotic process, as both mechanisms have been described. While oxidative stress can cause a reduction of mitochondrial membrane potential (Macho et al., 1999) probably via glutathione depletion (Macho et al., 1997), the opposite effect is also possible, i. e. that loss of mitochondrial membrane potential results in simultaneously increased ROS production as measured by DHE oxidation (Zamzami et al., 1995), which is an indicator of superoxide radicals. However, a hypothetical mechanism of ROS formation as a second step after disruption of mitochondrial membrane potential in PS1M146L-expressing cells is difficult to reconcile with some of our observations:

(i) Lymphocytes were analyzed by FACS measurement and gated according to size and granularity. Thus, only viable lymphocytes, but not apoptosing cells, were chosen for evaluation. As a loss in mitochondrial membrane potential leads to rapid induction of apoptosis, it is unlikely that the gated cells represent a large pool of apoptosing cells.

(ii) A decrease in mitochondrial membrane potential has been consistently reported to result in overproduction of superoxide radicals (Castedo et al., 1995; Zamzami et al., 1995), probably released from mitochondrial respiratory chain complexes (Turrens, 2003). However, we did not detect increased superoxide formation as evidenced by unaltered DHE oxidation.

For these reasons, it is unlikely that the increased DHR oxidation observed in splenic lymphocytes from PS1M146L transgenic mice is a secondary event due to loss of mitochondrial membrane integrity and apoptotic processes. Rather, increased ROS formation can be considered as the trigger for lymphocyte apoptosis in PS1M146L transgenic mice. Figure 4.3 summarizes the effects of mutant PS1 on the various parameters studied.

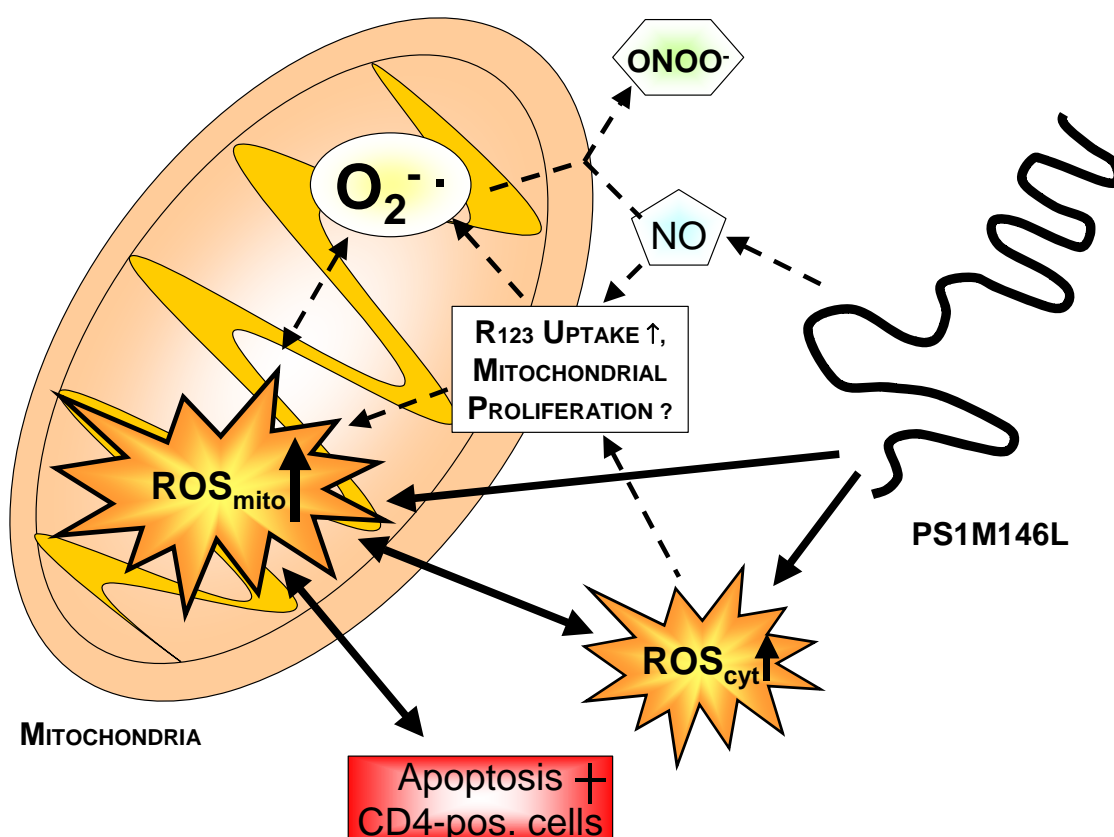


Figure 4.3: Alterations in ROS caused by PS1M146L in lymphocytes from PS1 transgenic mice.

Mutant presenilin 1 PS1M146L leads to elevation of mitochondrial and cytosolic ROS, but not superoxide radical production in lymphocytes from PS1 transgenic mice. Nitric oxide levels are marginally and not significantly increased. R123 staining of mitochondria is slightly elevated. Increased mitochondrial ROS levels in CD4-positive lymphocytes were correlated with levels of apoptosis.

Abbreviations: ROS_{mito}/ROS_{cyt} = mitochondrial/cytosolic reactive oxygen species.

Use of peripheral cells to study effects in brain tissue

A close interaction seems to exist between cells of the immune system and the central nervous system – either by modulation of the immune system by neuroendocrine signals or neurotransmitters (Hanisch et al., 1996; Carson and Sutcliffe, 1999), or *vice versa* by an impact of immune cells on the CNS (Hickey et al., 1991; Wekerle, 1993; Nitsch et al., 2004). Many neurological disorders are accompanied by changes in immune cell function (Schwarz et al., 2001; Fiszer, 2001) which led to studies of alterations in the function of lymphocytes and other peripheral cells as easily accessible models in neurodegenerative disease states (Eckert et al., 2001a; Gibson and Huang, 2002).

As many correlates between observations on brain tissue and peripheral cells of AD patients have been obtained in these studies, it seems feasible that changes in peripheral cells can be used to monitor alterations in brain tissue from AD patients. In an attempt

to extend these observations in humans to PS1 transgenic mice, a probable interaction between ROS levels measured in splenic lymphocytes and oxidative damage in brain tissue was analyzed. Correlation of DHR oxidation in lymphocytes with levels of HNE in brain tissue from each individual animal yielded a positive and significant linear relationship (Figure 3.18, page 106), suggesting that oxidative stress in brain tissue can be mirrored in peripheral cells. Of note, similar results have been obtained in the analysis of gender differences in mice, where lower levels of lipid peroxidation products in brain tissue from female compared to male mice were accompanied by lower DHR oxidation in peripheral lymphocytes (see 4.1.3, page 163). Hence the overall conclusion would be that similar oxidative stress-related mechanisms operate in cells from the central nervous system and in peripheral cells, adding further importance and relevance of the numerous studies conducted on peripheral cell models in neurodegenerative diseases so far (Eckert et al., 2001a; Cecchi et al., 2002; Gibson and Huang, 2002; Schindowski et al., 2003).

Putative sources of increased ROS production in PS1M146L transgenic mice

Under physiological conditions, mitochondria are considered to be the main intracellular source for ROS. In our experiments, ROS levels in PS1M146L cells were most profoundly increased in the mitochondrial compartment (42.1 % increase in DHR fluorescence relative to non-transgenic mice) as compared to the cytosol (20.5 % increase in DCFH-DA fluorescence), which could suggest a primary formation of ROS in mitochondria and then a subsequent leak into the cytosol. The more pronounced increase in mitochondrial as compared to cytosolic ROS levels could be explained by the relatively lower activity of GPx in mitochondria compared to cytosol (Timcenko-Youssef et al., 1985) – affording less protection against mitochondrial as compared to cytosolic ROS accumulation.

Mitochondrial ROS mostly arise from electron leakage from the respiratory chain, where the primary ROS formed is usually superoxide. However, superoxide formation was not increased in PS1M146L-bearing lymphocytes. Therefore, increased mitochondrial ROS could result from elevated flow of electrons along the mitochondrial respiratory chain with simultaneously rapid and very efficient removal of the generated superoxide by SOD enzymes. Although activities of antioxidant enzymes were not assayed in lymphocytes, enzyme activities are probably not impaired in these cells, as stimulation with oxidative stressors led to similar responses in non-transgenic and PS1-

expressing cells. Furthermore, no effect on antioxidant enzyme activities in brains from PS1 transgenic mice was detected. Hence, it is possible that increased ROS production in mitochondria with radical leak from the respiratory chain and rapid conversion to hydrogen peroxide results in the observed increased DHR oxidation, while increased superoxide radicals can not be detected.

Our results moreover suggest that mitochondria are not directly damaged in PS1M146L cells, as uptake of R123 into mitochondria was not impaired. In contrast, R123 staining was even elevated, suggesting that the number and mass of mitochondria in PS1M146L cells may be increased. An increase in the absolute number of mitochondria, but not mitochondrial impairment, could explain why overall DHR oxidation is increased in PS1M146L cells without a simultaneous rise in superoxide radical production. Mitochondrial proliferation has been described in response to nitric oxide (Nisoli et al., 2003), which could explain the observation of slightly but not significantly elevated NO levels (106.7 % relative to controls) and increased R123 uptake by mitochondria (107.2 % relative to controls). Alternatively, ROS can trigger lymphocyte activation (Roth and Droge, 1987), which is associated with an increased mitochondrial mass and R123 uptake (Darzynkiewicz et al., 1981; Ferlini et al., 1995). Therefore, the effects of increased mitochondrial ROS production in PS1M146L cells could be secondary to mitochondrial proliferation.

In summary, on the basis of our results it can not be decided whether increased ROS formation caused by mutant PS1M146L originates from mitochondrial or from cytosolic sources. Nevertheless, a direct and prominent damage of mitochondria seems unlikely as superoxide production is not elevated by the PS1 mutation and R123 uptake into mitochondria is not impaired.

Which mechanism can be responsible for increased ROS production in PS1M146L-expressing lymphocytes? Increased production of A β by presenilin mutations has been established as the main factor for presenilin toxicity. However, in murine lymphocytes, only rodent A β can be formed, and oxidative toxicity of rodent A β has not been investigated so far. Thus, although rodent A β is different from human A β in three amino acids that also have impact on the aggregation and oxidative properties of the peptide *in vitro* (Dyrks et al., 1993; Brzyska et al., 2001), it can not be excluded that the increased mitochondrial ROS formation is due to toxicity of endogenous murine A β . Furthermore, high levels of intracellular A β have been detected in neurons from mutant

PS1 transgenic mice, which also displayed neurodegeneration (Chui et al., 1999; Sadowski et al., 2004), raising the possibility that endogenous rodent A β may be toxic. This is supported by the finding that overexpression of rodent A β in transgenic mice led to neurodegeneration (LaFerla et al., 1995).

Apart from increased A β formation caused by mutant presenilins, several proteins and intracellular signaling cascades have been described to interact with presenilins, among these are Bcl-X_L (Passer et al., 1999) and Bcl-2 (Alberici et al., 1999). Bcl-2 and Bcl-X_L display antioxidant and antiapoptotic properties (Hockenbery et al., 1993; Kane et al., 1993). Thus, an impairment of Bcl-2 and Bcl-X_L function by presenilin mutations could explain the increased mitochondrial ROS production. However, whether these effects can operate in PS1M146L cells remains speculative as interactions with wildtype PS1 have been studied, but the effects of FAD PS1 mutations on these interactions are unknown.

Even more interesting are the effects of presenilins on intracellular calcium levels, as increased calcium levels can trigger several mechanisms of ROS formation, and mechanisms of interference of PS1 with calcium signaling pathways have been described, such as interactions with calsenilin (Buxbaum et al., 1998a), G₀-proteins, phospholipase C, IP₃-signaling and ryanoidine receptors (Smine et al., 1998; Chan et al., 2000; Popescu et al., 2004) and calpain (Maruyama et al., 2000). Additionally, the subcellular localization of presenilins in ER and colocalization with ryanoidine receptors (Chan et al., 2000) makes them a likely candidate for interference with ER calcium stores. Apart from direct effects of PS1M146L on intracellular calcium levels, disrupted calcium homeostasis in PS1M146L-expressing cells could also be mediated by oxidative stress, for example due to interference of ROS or HNE with phospholipase C activity (Rossi et al., 1988; Wang et al., 2001). HNE furthermore has been shown to impair calcium-ATPases (Siems et al., 2003), which may additionally contribute to increased intracellular calcium levels.

Numerous studies have established defective calcium signaling in cell culture (Guo et al., 1996; Leissring et al., 1999; Leissring et al., 2000) and transgenic mice bearing PS1 with FAD mutations (Begley et al., 1999; Guo et al., 1999b; Chan et al., 2000; Schneider et al., 2001b; Stutzmann et al., 2004). Deficits in intracellular calcium regulation have moreover been detected in peripheral cells from FAD patients bearing PS1 mutations and in sporadic AD patients (Eckert et al., 1994; Ito et al., 1994; Gibson

et al., 1996; Etcheberrigaray et al., 1998), suggesting a pathological role for disturbed calcium homeostasis as a final common pathway of both forms of AD. Furthermore, in an earlier study in our group, splenic lymphocytes from 3 months old PS1 transgenic mice bearing five mutations in PS1 showed unchanged basal levels of calcium, but increased calcium levels after mitogenic stimulation (Eckert et al., 2001b), which lends further support to findings of impaired calcium homeostasis as a contributor to toxic effects of mutant PS1. Of note, the animals studied were of young age (3 months). As we observed a profound effect of aging on ROS accumulation caused by the PS1M146L transgene, it is possible that the defect in calcium regulation becomes similarly impaired in aged mice already under unstimulated conditions, which can explain some of our findings of elevated basal ROS levels in aged PS1M146L transgenic mice.

Increased intracellular calcium levels would fit well into our observations that cytosolic as well as mitochondrial ROS levels are increased in PS1M146L transgenic mice, as calcium can stimulate ROS formation by a variety of pathways. Calcium can activate phospholipase A resulting in increased arachidonic acid cascade activation and ROS production from lipoxygenase and cyclooxygenase reactions (O'Donnell et al., 1995; Gunasekar et al., 1998). Furthermore, neuronal and endothelial nitric oxide synthases are activated by increased calcium concentrations resulting in increased formation of nitric oxide, which was also detected in our studies, albeit only to a weak extent. Although pronounced elevations in nitric oxide levels caused by mutant PS1 had been observed in cell culture transfection studies before (Guo et al., 1999a; Hashimoto et al., 2002), profoundly increased NO levels could not be detected in our system, suggesting that transfection experiments on cell culture can lead to much more exaggerated PS1 effects as compared to the analysis of transgenic mouse cells.

Finally, manifold interactions between calcium and mitochondria exist (Chakraborti et al., 1999; Berridge et al., 2000). As an example, increased intracellular calcium levels can be buffered by mitochondria (Rizzuto et al., 1993), which in turn results in stimulation of mitochondrial metabolism and increased ATP synthesis (Berridge et al., 2000) and is followed by increased ROS formation (Dyken, 1994; Kowaltowski et al., 1995). Increased mitochondrial metabolism is in good accordance with the observed elevated uptake of R123 into mitochondria and elevated mitochondrial ROS production without a simultaneous rise in superoxide production, as mitochondria are not directly damaged. In summary, all the effects we observed could be reconciled with increased intracellular calcium levels in cells bearing presenilin mutations (see Figure 4.4).

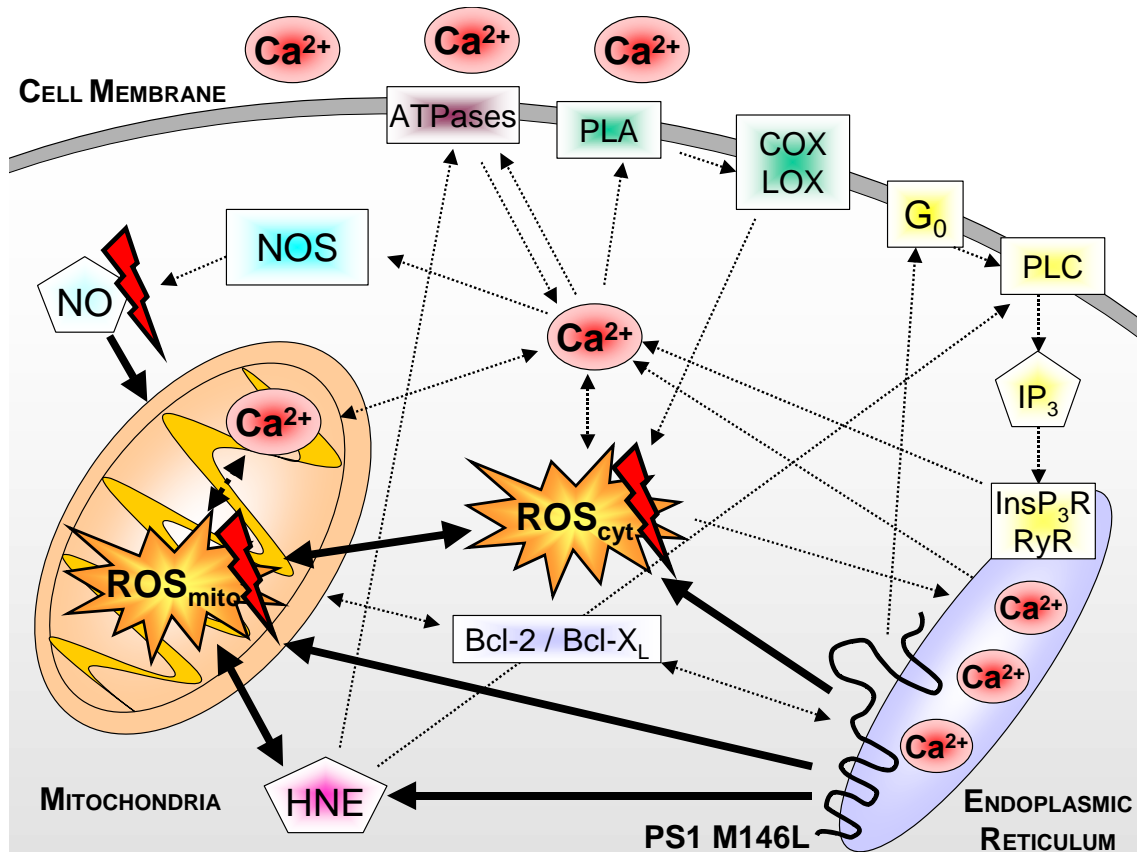


Figure 4.4: Putative mechanisms of increased ROS formation by PS1M146L in transgenic mice.

Elevation of mitochondrial and cytosolic ROS caused by PS1M146L can lead to lipid peroxidation, elevation of HNE levels and disruption of calcium homeostasis, linking further ROS production from cytosolic and mitochondrial sources in a vicious cycle. Straight arrows represent findings from this thesis, whereas dotted arrows represent putative interactions described by other groups. See text for further details.

Abbreviations: COX cyclooxygenase, HNE 4-Hydroxynonenal, InsP_3R inositol-triphosphate receptor, IP_3 inositol-1,4,5-triphosphate, LOX lipoxygenase, NOS nitric oxide synthase, NO nitric oxide, PLA phospholipase A, PLC phospholipase C, $\text{ROS}_{\text{mito}}/\text{ROS}_{\text{cyt}}$ mitochondrial/cytosolic reactive oxygen species, RyR ryanoidine receptor.

It is however difficult to speculate whether a disturbed calcium homeostasis is the primary effect resulting in increased ROS generation or whether – *vice versa* – increased ROS levels lead to disturbed calcium levels, as widely described (Macho et al., 1997; Chakraborti et al., 1999). Previous findings by our groups in young PS1M5 transgenic mice – where basal ROS levels but not basal calcium levels were elevated – suggest a primary role for ROS-induced calcium dyshomeostasis. Similarly, in a study on PS1 transfected PC12 cells (Guo et al., 1996), a PS1 mutation led to increased intracellular calcium levels, ROS production and apoptosis after $\text{A}\beta$ stimulation. The rise in intracellular calcium levels was obviously ROS-dependent, as vitamin E was able to completely prevent the rise in intracellular calcium and ROS levels. However, neither the L-type calcium channel antagonist nifedipin nor dantrolene, an inhibitor of calcium

release from ER stores, were able to completely inhibit ROS formation, suggesting that ROS can be produced independently from intracellular calcium levels in cells overexpressing mutant PS1. Thus, it is likely that increased ROS production caused by PS1 mutations is primarily responsible for calcium dyshomeostasis, which in turn can further exacerbate ROS formation in a vicious cycle. Therefore, the interactions between effects of presenilin mutations on ROS generation and intracellular calcium levels would be an interesting objective for future analysis in PS1 transgenic animals.

Summary of mutant PS1-induced effects

In summary, our results on changes in oxidative stress-parameters observed in brain tissue and peripheral cells support the hypothesis of oxidative toxicity caused by specifically the mutant PS1M146L when compared to PS1wt transgenic or non-transgenic mice.

The mechanism is probably related to increased ROS formation but not impaired detoxification, as we observed neither an impairment of antioxidant enzyme activities in brain tissue nor a different susceptibility towards oxidative stimuli in brain tissue and in lymphocytes. Moreover, oxidative toxicity of the PS1M146L mutation is evident only in aged animals, arguing for a combined effect of aging – the most important risk factor for the development of sporadic AD – and the FAD mutation on an elevated sensitivity towards ROS accumulation. Mild changes could be observed in brain tissue at the level of HNE (13.3 % increase relative to non-transgenic controls), while more pronounced effects could be detected when ROS levels were measured directly in splenic lymphocytes from PS1M146L transgenic animals. Of note, levels of mitochondrial ROS were 42.1 % and cytosolic ROS 20.5 % higher in PS1M146L cells relative to cells from non-transgenic littermate control mice, pointing to mitochondria as the main source of ROS. At the same time, superoxide production remained unchanged and R123 retention in mitochondria was not impaired, indicating that mitochondria are not profoundly damaged in living cells from PS1M146L mice. Hence, severe mitochondrial damage as a source of ROS in PS1M146L mice could be excluded from our experiments.

In addition, our observations emphasize a functional relevance for measurement of mitochondrial ROS levels in living cells, as DHR oxidation in CD4-positive lymphocytes was correlated with apoptosis levels. Furthermore, changes could be detected in both brain tissue as well as lymphocytes from PS1M146L mice, i.e. in

central as well as peripheral compartments, underscoring the suitability of lymphocyte studies in neurodegenerative diseases.

4.2.2 Effects of APP mutations on oxidative stress-related parameters

4.2.2.1 Analysis of PDGF-APP and PDGF-APP/PS1 double transgenic mice

A major confounding finding in this thesis is the fact that the single PS1 mutation in PS1M146L mice leads to elevated HNE levels in brain tissue, but no similar elevation is found in brains from double transgenic PDGF-APP/PS1 mice (Figure 3.9, page 96). Although the results need to be reproduced in another cohort, it is striking that HNE levels are not further increased but rather seem to be decreased in PDGF-APP/PS1 compared to PS1M146L transgenic mice, although differences are not statistically significant. This is unexpected since the effects of mutant PS1 on oxidative stress should at least be confirmed in the double transgenic mice and, furthermore, probably even exacerbated – based on numerous reports of oxidative toxicity for A β *in vitro* (Behl et al., 1994b; Butterfield et al., 1996).

A prominent accumulation of A β in the brains of double transgenic PDGF-APP/PS1 mice was demonstrated in Western blot and ELISA (Figure 3.31, page 123), and formation of A β plaques in the brains of these mice has been described previously (Blanchard et al., 2003), suggesting that merely the presence of human APP and/or the accumulation of A β in brains of transgenic mice during aging is insufficient to result in oxidative toxicity – at least oxidative damage was undetectable with our methods. It can only be speculated that either (i) APP expression and/or low formation of C99 peptide and A β do not exhibit prooxidative properties under the conditions in PDGF-APP or PDGF-APP/PS1 mouse brains, that (ii) the subcellular localization or the rate of APP expression, C99 and A β production determine oxidative pathology or (iii) that A β accumulation leads to adaptive responses in these transgenic mice that result in an overall efficient removal of ROS generated by A β – apart from enzymatic activities of Cu/Zn-SOD, GPx and GR, which were not affected (Figure 3.10, page 97).

The lack of a statistically significant difference in HNE levels in double transgenic PDGF-APP/PS1 mice is in contrast to increased HNE levels in single transgenic

PS1M146L mice. Is it possible that the additional expression of APP alters the effects of mutant PS1 in a way that abolishes oxidative toxicity? The overexpression of APP in brains from mice may lead to formation not only of A β , but also to increased formation of the N-terminal fragment sAPP α from endogenous α -secretase cleavage. As sAPP α exhibits neuroprotective properties (Guo et al., 1998), overexpression of APP in brains from PDGF-APP and PDGF-APP/PS1 transgenic mice may lead to increased formation of sAPP α thus counteracting oxidative toxicity caused by the PS1 mutation in single PS1M146L transgenic mice.

Alternatively, the toxicity or at least some part of the toxicity of mutant PS1 may be unrelated to formation of A β , since several effects of PS1 mutations on cell death and susceptibility towards apoptotic stimuli have been described (Chan et al., 2000; Kim et al., 2001). Furthermore, presenilins interact with various signaling cascades – apart from APP [reviewed in (Steiner and Haass, 2001)]. Moreover, the rate of increased A β production by different PS1 mutations is not related to the clinical onset of FAD in patients (Gomez-Isla et al., 1999; Lleo et al., 2004), suggesting that an increased production of A β may not be the only mechanism relevant for neurotoxic effects of presenilin mutations in AD pathogenesis. Overall it can be speculated that deficits in several intracellular signaling cascades in PS1M146L single transgenic mice can result in oxidative toxicity. Coexpression of APP in double transgenic PDGF-APP/PS1 mice may however lead to a significant degree of PS1 interaction with the overexpressed APP proteins (Waragai et al., 1997; Xia et al., 1997) or to a different subcellular localization caused by interaction of PS1 with APP (Cai et al., 2003). Thus, interaction with transgenic APP could intercept PS1 proteins from signaling routes which cause oxidative toxicity in single transgenic PS1M146L mice.

Conclusion from studies on PDGF-APP and PDGF-APP/PS1 transgenic mice

Taken together, neither the presence of A β and/or APP nor the deposition of A β plaques in brain tissue is sufficient for triggering oxidative stress pathology in PDGF-APP and PDGF-APP/PS1 transgenic mice.

4.2.2.2 Analysis of Thy1-APP transgenic mice***Oxidative stress-related parameters***

A very different picture emerged when transgenic mice bearing the 751 amino acid form of APP under the control of a neuron-specific murine Thy1-promotor were analyzed. In these mice, oxidative damage detected by elevated HNE content was found already at 3 months of age (115 % of non-transgenic controls), which was even more pronounced and significantly different from non-transgenic mice at 12 months of age (184 % of non-transgenic controls). Surprisingly, however, no difference was observed in 18 months old Thy1-APP transgenic mice (see Figure 3.19, page 109). Thus, elevation of HNE levels starts early during the life span of Thy1-APP transgenic mice, reaches a peak at mid age and declines to the levels of non-transgenic mice at high age. Furthermore, oxidative damage in this mouse model can be detected only with HNE but not with MDA – reminiscent of the results in mutant PS1 transgenic mice (see Figure 3.9, page 96) and AD patients (see Figure 3.39, page 133). Thus, the aldehydes seem to detect different kinds of oxidative damage, which will be discussed in more detail in sections 4.3.2.2, page 215 et seqq., and 4.4.2, page 229 et seqq.

Analysis of enzymatic antioxidant defense revealed that an impairment of Cu/Zn-SOD activity is present in Thy1-APP transgenic mice, which parallels the increased oxidative damage at the level of HNE: enzyme activity showed a tendency towards reduced levels in 3 months old Thy1-APP transgenic animals, was significantly reduced in 12 months old animals, but receded to the levels of non-transgenic mice in 18 months old Thy1-APP transgenic mice (Figure 3.20, page 110). Thus, impairment of Cu/Zn-SOD activity in young and middle-aged Thy1-APP transgenic mice may be causally related to increased HNE levels in brains from these mice. It may be that the upregulation of antioxidant enzymes during aging observed previously in C57BL/6J mice counteracts the effects of the transgenic APP on Cu/Zn-SOD activity in aged (18 months old) transgenic mice, hence reduced Cu/Zn-SOD activity and increased HNE levels can no longer be detected in this age group.

Although impairment of Cu/Zn-SOD activity may be causally responsible for increased basal lipid peroxidation levels in young and middle-aged Thy1-APP transgenic mice, the extent of *in vitro* stimulation of lipid peroxidation with ferric iron was not different between Thy1-APP transgenic and age-matched non-transgenic mice (Figure 3.21, page 111). These results suggest that reduced Cu/Zn-SOD activity is not a major contributor

to *in vitro* accumulation of ROS and formation of lipid peroxidation products after oxidative stimulation with ferric iron in this assay.

Previously made observations in brain tissue from non-transgenic C57BL/6J mice (see section 3.1.1.1) that levels of lipid peroxidation products decline whereas activities of antioxidant enzymes rise during aging could be reproduced in this cohort of mice (see Figure 3.19, page 109 for lipid peroxidation, and Figure 3.20, page 110 for enzyme activities). The effect just failed to reach statistical significance for Cu/Zn-SOD (ANOVA $p < 0.08$ affect of aging) and was not significant for GPx activity – as only male mice with inherently lower activity of GPx were studied in the 18 months old group precluding an evaluation of increased GPx activity with aging. GR activity was however significantly increased in aged mice (ANOVA $p < 0.0001$ effect of aging). Furthermore, gender differences (see 3.1.2.1) – i.e. that female mice exhibit lower levels of lipid peroxidation products and higher activity of GPx – could be reproduced in this cohort, which is independent of the Thy1-APP transgene (see Figure 3.22, page 112, and Figure 3.24, page 114).

However, a transgene-specific gender difference was detected in the onset of oxidative changes: at 3 months of age, female Thy1-APP transgenic mice showed increased HNE levels and reduced Cu/Zn-SOD activity compared to female non-transgenic mice, while male Thy1-APP transgenic mice at that age were not affected (Figure 3.22, page 112). Curiously, MDA levels were also elevated in 3 months old female Thy1-APP transgenic mice, however the extent of increase in MDA levels (107.8 % relative to non-transgenic controls) is much lower than that of HNE levels (135.7 % relative to non-transgenic controls) and thus seems of minor importance. At 12 months of age, both gender of Thy1-APP transgenic mice were affected to a similar extent by elevated HNE levels and impaired Cu/Zn-SOD activity compared to age- and gender-matched control animals (Figure 3.23, page 113). At 18 months of age, only male animals could be studied for reasons of availability, which precluded analysis of gender differences at that age. Furthermore, 12 months old female Thy1-APP transgenic mice displayed increased MDA formation after stimulation of lipid peroxidation with ferric iron *in vitro* compared to age-matched non-transgenic female mice (Figure 3.25, page 115). Hence, it can be concluded that female Thy1-APP transgenic mice do not only display changes in oxidative stress-related parameters earlier than male mice, but also that at mid-age female Thy1-APP transgenic mice react more sensitive towards stimulation of lipid peroxidation.

Expression of APP and formation of A β during aging

In an attempt to clarify some of our observations, we analyzed levels of APP expression and A β formation in brains of Thy1-APP transgenic mice during aging. Western blot analysis revealed that expression levels of the transgenic full-length APP protein remain relatively constant during aging and are independent of gender (Figure 3.29, page 120). However, levels of A β and C99 peptide accumulate with aging, and a gender-disparate higher formation of A β and C99 peptide was observed in brains from female transgenic mice. As both A β and C99 levels were increased in brains from female mice, the gender difference is probably caused by altered β -secretase cleavage of APP but not by γ -secretase cleavage nor by altered accumulation of the A β peptide. The differences in insoluble A β levels observed in Western blot were confirmed by ELISA analysis of the soluble A β_{1-40} pools, where female transgenic mice consistently displayed approximately 30 % higher A β levels compared to male mice. Of note, this gender difference in A β production is conserved during aging, despite the high rate of accumulation of A β with aging in this mouse model.

As female mice show higher β -amyloidogenic cleavage of APP and display increased HNE levels and reduced Cu/Zn-SOD activity earlier than male mice, the observed changes in oxidative stress-related parameters seem to be closely correlated with the presence of C99 peptide and/or A β . The gender-disparate increased levels of A β in 12 months old female Thy1-APP transgenic mice combined with reduced Cu/Zn-SOD activity could furthermore be related to the higher susceptibility towards *in vitro* stimulation of lipid peroxidation of female transgenic mice at this age group. It is however difficult to explain the complete lack of oxidative damage and/or reduced Cu/Zn-SOD activity in 18 months old Thy1-APP transgenic mice. It may be that adaptive changes take place during aging – for example, we had observed an upregulation of Cu/Zn-SOD activity and other antioxidant enzymes and a reduction in the levels of lipid peroxidation products in non-transgenic mice during aging (see 3.1.1.1). Hence, these adaptive processes in aged mouse brains may counteract the deleterious effects of the APP transgene. Alternatively, an altered subcellular distribution of A β in aged mouse brains could contribute to this effect, which will be discussed in more detail on page 195 et seqq.

Summary and discussion of oxidative stress-related changes in Thy1-APP transgenic mice

In summary, our results show oxidative stress pathology in Thy1-APP transgenic mice as measured by increased levels of the lipid peroxidation product HNE, which is probably related to impaired Cu/Zn-SOD activity. Furthermore, oxidative pathology is first observed in brains from 3 months old female Thy1-APP mice, which display higher formation of A β and C99 than male mice, and notably before the onset of plaque deposition. Our results underscore the role of A β and/or C99 accumulation in oxidative stress pathology and suggest a direct and causal relationship between increased A β and/or C99 levels, reduced Cu/Zn-SOD activity and oxidative damage.

Impaired Cu/Zn-SOD activity in Thy1-APP mice can lead to increased formation of lipid peroxidation products

From our results on aging mouse brain tissue and on gender differences in enzymatic antioxidant defense and levels of lipid peroxidation products, it can be concluded that the activities of antioxidant enzymes are a major contributor to the protection against free radical attack on cellular membranes and formation of lipid peroxidation products. This is supported by two observations: (i) aged animals exhibit higher levels of Cu/Zn-SOD, GPx and GR and correspondingly lower levels of lipid peroxidation products and (ii) female mice in general have higher activities of GPx and correspondingly lower levels of lipid peroxidation products. *Vice versa*, reduced activity of Cu/Zn-SOD in Thy1-APP transgenic mice supposedly favours the accumulation of superoxide radicals that lead to oxidative damage resulting in increased HNE levels in brains from these mice. Exactly how superoxide radicals can mediate lipid peroxidation processes is not completely understood in detail, but it is conceivable that lipid peroxidation reactions are initiated through hydroxyl radicals generated via Haber-Weiss and Fenton reactions (Halliwell and Gutteridge, 1999) or that not superoxide itself but its protonated form, the perhydroxyl radical, can initiate lipid peroxidation reactions (Aikens and Dix, 1991). Another superoxide-dependent mechanism of lipid peroxidation has been attributed to increased formation of peroxynitrite from nitric oxide and superoxide (Radi et al., 1991). Addition of superoxide dismutase to *in vitro* systems of lipid peroxidation studies prevented lipid peroxidation (Tien et al., 1981; Gutteridge et al., 1983; Gutteridge, 1984). Consequently, superoxide dismutase/catalase mimetics have been shown to protect against oxidative damage and cognitive deficits in a murine model of

aging (Liu et al., 2003), and mice transgenic for Cu/Zn-SOD exhibit lower levels of the lipid peroxidation product HNE (Bonnes-Taourel et al., 1993). It is therefore conceivable that reduced activity of Cu/Zn-SOD in Thy1-APP mice causes increased formation of HNE.

Additionally, Cu/Zn-SOD proved to be directly protective against APP or A β toxicity in a variety of paradigms: endothelial deficits in APP transgenic mice could be reversed by either exogenous application of Cu/Zn-SOD or coexpression of Cu/Zn-SOD (Iadecola et al., 1999). Furthermore, overexpression of Cu/Zn-SOD in SHSY5Y cells protected against A β -induced cell death (Celsi et al., 2004), and Cu/Zn-SOD expression in APP transgenic mice rescued embryonic lethality due to overexpression of APP (Carlson et al., 1997). Thus, pathogenic events related to the overexpression of APP and/or A β accumulation may be mediated by increased superoxide radical formation and can be counteracted by SOD enzymes.

Overexpression of APP in transgenic mice leads to reduced Cu/Zn-SOD activity

A primary factor that can lead to impaired activity of Cu/Zn-SOD in Thy1-APP mice probably resides in the interaction of APP or A β with metal ions. Full-length APP as well as A β can bind copper ions *in vitro* (Hesse et al., 1994; Atwood et al., 1998), and these findings may be involved in regulation of copper homeostasis by APP *in vivo*: APP knockout mice show increased levels of copper ions in their brains (White et al., 1999), whereas APP overexpression in different transgenic mouse models leads to reduced levels of copper (Maynard et al., 2002; Phinney et al., 2003). In an independent study of still another APP transgenic mouse model, APP23 transgenic mice, we observed reduced copper levels as well as reduced Cu/Zn-SOD activity [see Figure 3.26, page 116, and (Bayer et al., 2003)], suggesting that overexpression of APP consistently leads to loss of copper ions from the brains of APP transgenic mice resulting in detectable impairment of Cu/Zn-SOD activity. Furthermore, supplementation of copper ions in the drinking water of APP23 mice augmented Cu/Zn-SOD activity towards the levels of non-transgenic control animals and also led to a significant reduction in A β levels (Bayer et al., 2003). Therefore, reduced availability of copper ions in the brains of APP transgenic mice is likely involved in impaired enzyme activity and may moreover affect A β formation and/or accumulation.

Although we can not exclude direct effects of APP on Cu/Zn-SOD activity, in the study by Maynard and coworkers (Maynard et al., 2002), a copper deficiency was evident not

only in mice overexpressing full-length APP but also in mice transgenic for the C100 fragment representing the 100 carboxy-terminal amino acids of APP. Similarly, our results suggest that increased levels of C99 or A β are responsible for reduced Cu/Zn-SOD activity since (i) we did not observe impaired enzyme activity in PDGF-APP-expressing mice despite similar overexpression of full-length APP and (ii) 3 months old female Thy1-APP mice, which exhibit higher levels of C99 and A β , show impaired enzyme activity while male mice of the same age do not, despite similar expression levels of full-length APP751.

So far, only one study analyzing antioxidant defense in human post mortem brain specimen from familial AD patients bearing APP mutations has been published. Bogdanovic and coworkers analyzed activities of antioxidant enzymes and found that activity of Cu/Zn-SOD showed a trend towards reduced activity in familial AD patients with the Swedish APP mutation compared to sporadic AD patients (Bogdanovic et al., 2001). This study provides important evidence that mutant APP and/or increased β -amyloidogenic processing of APP accompanied by increased formation of A β can lead to impairment of Cu/Zn-SOD activity also in humans.

Gender differences in transgenic mouse models of AD and human sporadic AD

In several different APP transgenic mouse models, female mice have been reported to exhibit increased or accelerated plaque formation compared to male transgenic mice: in Tg2576 mice (Callahan et al., 2001; Lee et al., 2002), in APP23 mice (Bayer et al., 2003) as well as in double transgenic PDAPP/PS1A246E mice (Wang et al., 2003). Despite the differences in length of APP, number of APP mutations and promoters used to drive transgene expression as well as divergent genetic background between the different mouse models, female mice consistently show elevated formation of A β and plaque deposition. Therefore it is likely that these observations are not merely artefacts of some special property of a single mouse model but rather that sex and/or unknown endocrine factors modulate APP processing towards the β -amyloidogenic pathway in general.

In contrast to Thy1-APP mice, we could not observe gender differences in A β formation in PDGF-APP or PDGF-APP/PS1 transgenic mice. The coexpression of mutant PS1 does not abolish gender differences seen in single transgenic APP-expressing mice, as Wang and colleagues have reported gender differences in APP and PS1 double transgenic mice (Wang et al., 2003). It may rather be that gender differences in A β

levels are undetectable in PDGF-APP/PS1 mice due to the relatively slow accumulation of A β and hardly detectable levels of C99 peptide. Alternatively, the gender differences in Thy1-APP transgenic mice could be due to a saturation effect of pathways that metabolize the C99 peptide and A β in female mice. Such a saturation effect would only be observed in Thy1-APP mice where high levels of C99 and A β can accumulate, but not in PDGF-APP and PDGF-APP/PS1 mice where low C99 and A β levels prevent their accumulation.

With respect to epidemiological studies reporting a higher prevalence of AD in females and corresponding gender differences in A β accumulation in APP transgenic mouse models, the effects of estrogen on APP processing have gained considerable attention and were studied in cell culture (Jaffe et al., 1994; Xu et al., 1998) and in APP-transgenic mouse models (Zheng et al., 2002; Levin-Allerhand et al., 2002). The general conclusions drawn from these studies were that estrogen treatment can decrease formation of A β . This is in contrast to our observations that female mice – where estrogen levels are supposed to be higher than in male mice – show higher A β formation compared to male mice. Later studies analyzing the modulation of A β by estrogen in mouse models have however produced conflicting results: Heikkinen and colleagues could not detect a beneficial effect of estrogen supplementation against accumulation of A β in APP and PS1 double transgenic mice (Heikkinen et al., 2004). Moreover, it remains unexplained why 17- α -estradiol with no affinity for estrogen receptors was even more effective in protecting against A β formation than the natural ligand 17- β -estradiol in the study by Levin-Allerhand and colleagues (2002). Hence, from these results and our observations it seems unlikely that endogenous levels of estrogens in transgenic mice reduce β -amyloidogenic processing of APP to a relevant extent *in vivo*. In accordance with this possibility, luteinizing hormone has been recently shown to increase beta-amyloidogenic cleavage of APP in cell culture (Bowen et al., 2004), hence it is possible that not only gonadal but also pituitary hormones can affect AD-relevant processes.

Although direct evidence is still controversial that – in the sporadic form of AD – women show higher β -amyloidogenic cleavage of APP (Sandberg et al., 2001), increased A β load in elderly women (Corder et al., 2004) and in female AD patients (Johnson et al., 1998) as well as a gender difference in the distribution of plaques (Kraszpulski et al., 2001) have been reported, providing first evidence that gender

differences in APP processing observed in mouse models might be relevant for the disease in humans.

Oxidative stress occurs in Thy1-APP mice independently of plaque formation

In our study, we were able to demonstrate that oxidative damage is present already in 3 months old female Thy1-APP mice. These mice develop first plaques at the age of 6 months, thus, oxidative damage is an early event and occurs independent of plaque formation. In other studies on different APP transgenic mice, markers for oxidative damage have been consistently detected only in Tg2576 mice (Pappolla et al., 1998; Smith et al., 1998; Pratico et al., 2001; Matsuoka et al., 2001a). However, mitochondrial alterations have also been identified in other mouse models: in Tg2576 (Reddy et al., 2004), in Thy1-APP (Blanchard et al., 2003) and in YAC-APP transgenic mice (Aliev et al., 2003). Furthermore, markers for nitrosative stress were increased in APP23 mice (Luth et al., 2001). While initial findings used immunohistochemical methods and detected HNE-modified proteins associated with A β plaques, it was thought that oxidative stress is caused by the deposition of A β plaques (Pappolla et al., 1998; Smith et al., 1998). However, a later report by Pratico and coworkers reversed these assumptions by providing evidence that isoprostanes as increased markers of oxidative damage can be detected already in 8 months old Tg2576 mice, which is 2-3 months prior to the onset of plaque deposition in these mice (Pratico et al., 2001). We were able to detect oxidative damage even earlier, already at an age of 3 months in female Thy1-APP transgenic mice which display onset of plaque formation from an age of 6 months. These findings support the notion that oxidative stress is an early pathogenic event in APP transgenic mice and occurs independently of plaque formation.

The mechanisms of APP cleavage, A β trafficking and deposition in APP transgenic mice have been studied in detail (LaFerla et al., 1996; Li et al., 1999; Wirths et al., 2001; Wirths et al., 2002; Blanchard et al., 2003). Prior to plaque deposition, A β was detected intracellularly, while in aged animals with severe plaque deposition, intracellular A β staining was no longer present. Similar findings were obtained in human brain autopsy samples, where A β was found intracellularly before the formation of plaques (LaFerla et al., 1997; Gouras et al., 2000). The intracellular pool of A β might play a pivotal role in oxidative stress toxicity, as it was shown that early intraneuronal A β accumulation in Thy1-APP/PS1 transgenic mice was associated with markers for mitochondrial dysfunction like cytochrome c and bax proteins (Blanchard et al., 2003).

Moreover, high intraneuronal A β may also contribute to the reported neurodegeneration in these animals, as neuron loss exceeded the areas of A β plaque formation (Schmitz et al., 2004). A later reduction of intracellular A β levels in aged animals (Wirths et al., 2002) could also explain why we could no longer detect increased HNE levels in 18 months old Thy1-APP transgenic mice. Hence it can be hypothesized whether oxidative stress is caused by intracellular A β accumulation in Thy1-APP transgenic mice.

4.2.2.3 Reduced activity of Cu/Zn-SOD in APP23 transgenic mice

As already observed previously in Thy1-APP transgenic mice, activity of the antioxidant enzyme Cu/Zn-SOD was similarly reduced in APP23 transgenic mice [see Figure 3.26, page 116, and (Bayer et al., 2003)]. Hence, the observation that overexpression of APP with familial AD mutations in transgenic mice leads to impairment of antioxidant defence by the enzyme Cu/Zn-SOD could be independently reproduced. Thus, our results suggest that failure of antioxidant systems to intercept free radicals can cause oxidative damage in APP transgenic mouse models, and the identification of reduced Cu/Zn-SOD activity represents a novel mechanism to explain oxidative toxicity triggered by mutant APP. Moreover, similar mechanisms of APP toxicity may operate in human carriers of APP mutations, as total SOD activity showed a trend towards reduced activity in a study of brain tissue from familial AD patients with the Swedish APP mutation compared to sporadic AD patients (Bogdanovic et al., 2001).

The relative extent of reduction of Cu/Zn-SOD activity in Thy1-APP and APP23 transgenic mouse brains was slightly different (see Table 4.4).

Although transgenic mouse brains were only available at different ages, it is obvious that the relative reduction in Cu/Zn-SOD activity is more pronounced in APP23 mice. Furthermore, this reduced activity was still detectable in 21 months old APP23 mice, while 18 months old Thy1-APP mice showed no difference relative to non-transgenic age-matched controls.

Table 4.4: Relative reduction of Cu/Zn-SOD activity in Thy1-APP and APP23 transgenic mouse brains.

age	Thy1-APP	APP23
3 months	6.58	n.a.
12 months	8.83	n.a.
15 months	n.a.	20.1
18 months	-0.35	n.a.
21 months	n.a.	16.17

Values represent the percentage of reduction in Cu/Zn-SOD activity relative to age- and gender-matched non-transgenic control animals.

n.a. = not available

Both these observations may be explained by a slight difference between the two mouse models, as the Thy1-APP mouse model contains an additional familial AD mutation. The two APP transgenic mouse models express the 751 amino acid form of human APP under the control of the murine Thy1 promotor. However, in APP23 transgenic mice only the Swedish double mutation is present, whereas the APP transgene in Thy1-APP mice contains the Swedish double and the London mutations. The London mutation V717I leads to increased formation of longer $A\beta_{1-42}$ and $A\beta_{1-43}$ species (Suzuki et al., 1994; Scheuner et al., 1996; Eckman et al., 1997), which have a higher tendency to aggregate, accumulate and form $A\beta$ plaques in brain tissue (Iwatsubo et al., 1994). As $A\beta$ has been described to bind copper ions (Atwood et al., 1998), with an especially high binding affinity of the $A\beta_{1-42}$ species (Atwood et al., 2000), it seems possible that accumulation of the longer $A\beta_{1-42}$ species in brains from Thy1-APP transgenic mice during aging leads to a higher binding and retention of copper ions in brains of these mice compared to APP23 transgenic mice. Hence, the relative reduction in activity of Cu/Zn-SOD would not be as pronounced as in APP23 transgenic mice and would be ameliorated with aging.

A second contributing factor may be that the relative formation of $A\beta_{1-40}$ species would be higher in APP23 mice with only the Swedish mutations compared to Thy1-APP transgenic mice with the extra London mutation which favours $A\beta_{1-42}$ formation. If copper ions are transported out of the brains of APP transgenic mice in a soluble form bound to $A\beta$ species, a relatively higher amount of soluble $A\beta_{1-40}$ in brains from APP23 transgenic mice would result in a higher export of copper ions out of the brain of these mice. Consistent with this hypothesis, the APP23 transgenic mouse model was reported

to display high accumulation of vascular amyloid deposition relative to other APP transgenics (Sturchler-Pierrat and Staufenbiel, 2000; Kalback et al., 2002; Mueggler et al., 2002), suggesting that a high proportion of A β species in this mouse model is transported from neurons, the site of A β production, to the brain vasculature. Hence, a putatively higher loss of copper via a mechanism of A β excretion from the brains of APP23 transgenic mice may result in the higher degree of Cu/Zn-SOD activity reduction in this mouse model. However, this remains speculation, as the APP23 (generated by Novartis Pharma) and the Thy1-APP (generated by Aventis Pharma) transgenic mouse models have not been compared in a direct study analysing A β formation and deposition so far.

Conclusion

In summary, although the extent of impairment of Cu/Zn-SOD activity in APP23 transgenic mice was more pronounced than in Thy1-APP transgenics, our findings of reduced Cu/Zn-SOD activity in APP transgenic mice could be independently reproduced and thus identify a novel mechanism for oxidative toxicity caused by mutant APP.

4.2.3 Comparison of different AD transgenic mouse models

In summary, our results show divergent oxidative stress pathology in different transgenic mouse models of Alzheimer's disease. While MDA seems to be an inappropriate marker for studying oxidative damage associated with FAD mutations, increased levels of HNE could be detected in mutant PS1 transgenic and Thy1-APP transgenic mice. Interestingly, no changes were observed in PDGF-APP and PDGF-APP/PS1 transgenic mice.

What are the major differences between PDGF-APP and Thy1-APP transgenic mice that can explain the lack of oxidative damage in PDGF-APP transgenic mice?

As shown by Western blot analysis of APP expression, levels of the full-length protein are rather similar in PDGF-APP and Thy1-APP transgenic mice (Figure 3.29, page 120). Thus, not merely the amount of full-length APP can serve to explain discrepancies between the two mouse models. The most striking differences between the two models therefore reside in the following factors:

(i) *The type of APP expressed*: the 695 amino acid form expressed in PDGF-APP mice with the Swedish, Dutch and London mutations versus the 751 amino acid form expressed in Thy1-APP transgenic mice with the Swedish and London mutations. The 751 amino acid form of APP contains a Kunitz-type serine protease inhibitor (KPI) domain and has been reported to yield higher levels of A β by increasing β -secretase cleavage of APP compared to the 695 amino acid form (Ho et al., 1996). This difference may explain the higher rate of C99 formation from β -secretase cleavage and also higher A β accumulation in Thy1-APP mice expressing APP751 (see Figure 3.29, page 120, Figure 3.31, page 123, and Figure 3.32, page 125). Of note, neurodegeneration and cognitive deficits have also been identified in mice expressing only the C-terminus of APP, suggesting that neurotoxicity can be exerted also by the C99 peptide (Kammesheidt et al., 1992; Oster-Granite et al., 1996; Neve et al., 1996; Nalbantoglu et al., 1997). Hence, the relatively low formation of C99 may explain why PDGF/APP and/or PDGF-APP/PS1 transgenic mice did not show any changes in oxidative stress-related parameters – despite the presence of A β and A β plaques – compared to Thy1-APP transgenic mice, which display high C99 formation. Interestingly, APP isoforms containing KPI domains may moreover be especially relevant for pathogenic events in AD brains, as a relative increase of KPI-containing APP isoforms over the APP695 isoform has been detected in brain tissue from AD patients (Tanaka et al., 1992; Zhan et al., 1995; Rockenstein et al., 1995; Moir et al., 1998; Preece et al., 2004). Moreover, similar to Thy1-APP transgenic mice, increased levels of the C-terminal fragment of APP have been detected in AD brains (Evin et al., 2003), supporting an important role of this protein in AD pathogenesis.

(ii) *The different FAD mutations present in APP*: PDGF-APP mice express APP with the Swedish, Dutch and London mutations, whereas Thy1-APP transgenic mice express APP bearing only the Swedish and London mutations. While the Swedish mutations – located at the β -secretase cleavage site – lead to increased β -amyloidogenic cleavage of APP resulting in elevated levels of total A β species (Citron et al., 1992; Mullan et al., 1992), the London mutation – located near the γ -secretase cleavage site – leads to a specific elevation of A β ₁₋₄₂ (Suzuki et al., 1994; Scheuner et al., 1996; Eckman et al., 1997). The Dutch mutation, localized at the α -secretase cleavage site and within the A β sequence, alters proteolytical processing of APP towards formation of lower levels of sAPP α (Haass et al., 1994) and alters fibril formation of A β species resulting in

accelerated A β aggregation (Levy et al., 1990; Wisniewski et al., 1991; Fraser et al., 1992; Clements et al., 1993). Thus, the additional presence of the Dutch mutation in PDGF-APP relative to Thy1-APP transgenic mice may result in altered aggregation properties of A β species in these animals, which probably also affects the localization in brain tissue, as a large pool of vascular A β deposits can be found in human carriers of the Dutch mutation (Levy et al., 1990) and in APPDutch transgenic mice (Herzig et al., 2004). Therefore, different aggregation properties, altered intra- or extracellular concentrations or spatial distribution of A β could have an outcome on the presence of oxidative stress pathology in different APP transgenic mice.

(iii) *The cellular or subcellular localization of APP*, as the PDGF promotor leads to only moderate and more homogenous expression in mouse brains while the Thy1-promotor leads to especially high expression levels in some neurons (Blanchard et al., 2003). As β -secretase activity was demonstrated to be high in neurons but low in astrocytes in APP transgenic mice (Zhao et al., 1996), high neuronal expression of APP in Thy1-APP mouse brains can explain the higher levels of C99 peptide derived from β -secretase cleavage of APP in these animals compared to PDGF-APP transgenic mice. Therefore, high neuronal expression and increased β -secretase processing of APP may be necessary for stimulation of oxidative stress toxicity in APP transgenic mouse models.

(iv) *The rate of A β accumulation*, which occurs much faster in Thy1-APP transgenic mice and leads to formation of larger and more widespread plaques (Blanchard et al., 2003).

Thus the overall impression would be that high formation of A β and/or C99 peptide, which is facilitated in Thy1-APP transgenic mice bearing APP with the KPI insert, coupled with high neuronal expression due to the Thy1-promotor and accompanied by a quick rate of A β accumulation leads to oxidative damage, while less pronounced A β accumulation caused by a more homogenous but only moderate expression of APP in neuronal cells from PDGF-APP transgenic mice does not elicit oxidative damage. The elucidation of the mechanisms of A β formation, the varying A β expression levels and the subcellular localization in different APP transgenic mouse brains may shed some more light on the exact molecular mechanisms that are causative for triggering oxidative stress in the different transgenic mouse models for AD.

Summary of observations in FAD transgenic mouse models

In summary, our results lead to the following conclusions:

(i) In PS1 transgenic mice, enzymatic antioxidant defense was not impaired suggesting that oxidative stress in these mice does not stem from impaired ROS detoxification but rather from increased ROS formation, which may be caused by elevated levels of endogenous mouse A β or by mechanisms unrelated to A β formation. Importantly, elevated ROS levels in cells from PS1M146L transgenic mice were only detected in aged animals (19-22 months old), suggesting that the aging process increases the vulnerability towards formation of ROS. This would be consistent with our previous results on C57BL/6J mice where aging causes a compensatory upregulation of activities of antioxidant enzymes, which occurs probably in response to elevated ROS formation.

(ii) In Thy1-APP transgenic mice, increased HNE levels and simultaneously reduced Cu/Zn-SOD activity could be detected. Moreover, the finding of reduced Cu/Zn-SOD activity could be independently reproduced in another APP transgenic mouse model, APP23 mice, suggesting that impairment of antioxidant defence via Cu/Zn-SOD caused by mutant APP is a common mechanism for oxidative toxicity in APP transgenic mouse models. Furthermore, a gender effect was observed: changes in these oxidative parameters were first detected in brains from 3 months old female Thy1-APP mice, which is notably before the onset of plaque deposition (Blanchard et al., 2003), and female mice display higher formation of A β and C99 than male mice. Our results underscore the role of A β and/or C99 accumulation, but not plaque formation, for triggering oxidative stress pathology and suggest a direct and causal relationship between increased A β and/or C99 levels, reduced Cu/Zn-SOD activity and oxidative damage. Interestingly, oxidative damage and impaired Cu/Zn-SOD activity were no longer detectable in aged Thy1-APP transgenic mice (18 months old), suggesting that the upregulation of activities of antioxidant enzymes during aging - which had been observed previously in C57BL/6J mice - can counteract deleterious effects of mutant APP in these mice.

(iii) The fact that plaque deposition is of minor importance for oxidative stress pathology is additionally supported by our findings that no alterations in oxidative stress-related parameters were observed in double transgenic PDGF-APP/PS1 mice, despite the presence of plaques in brains from these animals from an age of 6 months

(Blanchard et al., 2003). Reports from various other groups suggest that rather the intracellular accumulation of A β may play a pivotal role in neurotoxicity (LaFerla et al., 1997; Chui et al., 1999; Takahashi et al., 2002; Oddo et al., 2003).

Figure 4.5 summarizes the effects on oxidative stress-related parameters and putative mechanisms for oxidative toxicity in different AD transgenic mouse models studied in the course of this thesis.

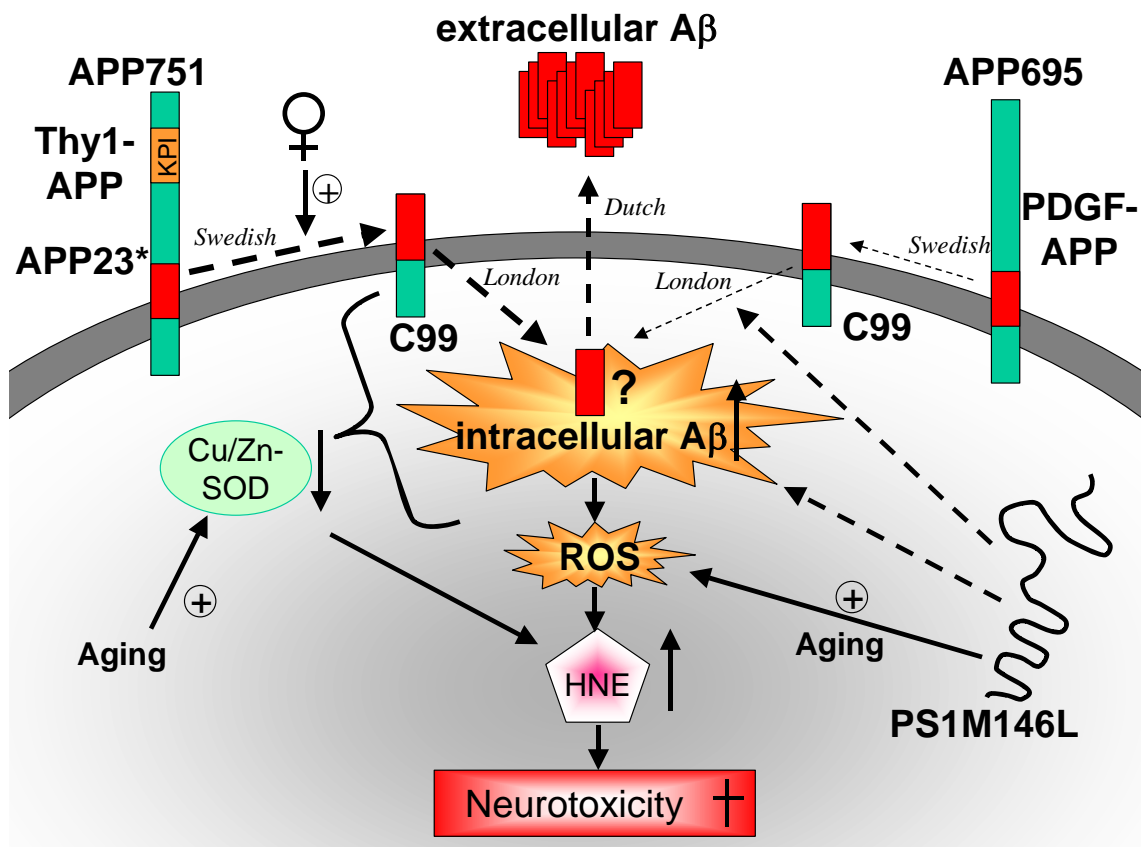


Figure 4.5: Summary of oxidative stress-related alterations observed in different AD transgenic mouse models and putative mechanisms for oxidative toxicity.

In Thy1-APP transgenic mice, high formation of C99 and/or A β , which is additionally increased in female transgenic mice, leads to impairment of Cu/Zn-SOD activity and elevation of HNE levels. This effect can be no longer observed in aged animals, as aging induces a compensatory rise in Cu/Zn-SOD activity. Reduced activity of Cu/Zn-SOD was also detectable in APP23 mice. Oxidative damage and reduced Cu/Zn-SOD activity were however not detectable in PDGF-APP or PDGF-APP/PS1 transgenic mice – despite the deposition of A β plaques in PDGF-APP/PS1 transgenic mice – suggesting that slow accumulation of A β or extracellular deposition do not cause oxidative damage. In contrast, a putative accumulation of intracellular A β may be responsible for oxidative toxicity. Increased HNE levels were also detected in mutant PS1 transgenic mice. In this mouse model, aging facilitates mechanisms of increased ROS formation.

Text in italics represents the respective familial AD mutations present in the different APP-transgenic mouse models. See text for further details.

* Only Cu/Zn-SOD activity was studied in APP23 mice.

In summary, oxidative damage is a feature of some, but not all transgenic mouse models. Furthermore, the mechanisms of origin of oxidative stress are heterogenous between PS1 and APP mutations: the PS1M146L mutation increases the formation of ROS but does seemingly not impair antioxidant defense, and this effect is more pronounced in aged animals (19-22 months old), suggesting that aging increases the vulnerability towards ROS formation. In contrast, mutant APP in Thy1-APP transgenic mice provokes oxidative damage by an impairment of Cu/Zn-SOD activity. In an independent study in another APP transgenic mouse model, APP23 mice, activity of Cu/Zn-SOD was similarly reduced. Hence, diminished antioxidant activity of the enzyme Cu/Zn-SOD represents a novel mechanism to explain oxidative toxicity of mutant APP in mouse models.

In conclusion, different mechanisms, i.e. increased ROS production triggered by PS1 mutations or impaired antioxidant activity of Cu/Zn-SOD caused by increased β -amyloidogenic processing of APP in mutant APP transgenic mice, can consistently lead to elevated levels of oxidative stress. Hence, our results support the hypothesis that oxidative damage constitutes a common pathway for neurodegeneration in familial AD patients carrying different FAD mutations.

4.3 Changes in oxidative stress-related parameters in sporadic AD patients

Although the discovery of familial AD mutations led to valuable insights into pathogenic mechanisms – mostly related to excessive formation of A β – and also provided the basis for generation of transgenic mouse models for AD, the vast majority of AD patients suffer from the sporadic form of the disease. It is therefore essential to prove that any pathogenic event identified in familial AD cases or transgenic models can be transferred to the situation in sporadic cases. Hence, we furthermore used post mortem brain tissue from sporadic AD patients and age-matched controls for analysis of lipid peroxidation products MDA and HNE as markers of oxidative damage and enzymatic antioxidant defense by Cu/Zn-SOD, GPx and GR. In a first set of samples, brain tissue from four different brain regions was employed including three different cortical brain regions and the cerebellum. This allowed for a broad screening of oxidative stress-induced alterations throughout various regions of the diseased brain. The results from this study prompted us to conduct a second set of experiments on a larger cohort of tissue samples from temporal cortex, as this was the brain region where changes were most pronounced in the first set of samples. In this second cohort, neuropathological data as well as a clinical assessment of dementia severity were available, which could be correlated with the biochemical parameters, providing important insights into putative causal relationships between oxidative stress-related parameters, sporadic AD risk factors and the clinical progression of the disease.

4.3.1 Cohort #1

In the first set of samples, a total number of 11 AD patients (7 female, 4 male) and 10 age-matched controls (6 female, 4 male) were studied. Brain tissue samples were obtained from 4 different brain regions: frontal cortex, parietal cortex, temporal cortex and cerebellum. These cortical regions are differently affected by the disease in the order of temporal > frontal > parietal cortex (Delacourte et al., 1999), while cerebellar functions are not impaired by the AD process. The samples were chosen to be matched as closely as possible for mean age at death and post mortem interval. As stated in 2.1.8.1 (page 56 et seq.), the distribution of Apo E4 alleles was higher in AD patients compared to controls, reflecting the well-known epidemiological association of the E4 allele with the risk to develop AD.

4.3.1.1 Upregulation of antioxidant defense prevents oxidative damage

We observed no differences in basal levels of lipid peroxidation products MDA or HNE between AD samples and controls (see Figure 3.33, page 127). Although the mean HNE levels were slightly increased in AD patients relative to controls in every brain region, the difference was not statistically significant due to the large variation of values. However, there was a trend towards lower HNE levels in control and AD samples from cerebellum (ANOVA $p = 0.08$). As cerebellar functions are not compromised by the disease, it can be speculated whether reduced HNE levels constitute one of the factors that contribute to a selective resistance of cerebellar neurons against degeneration.

Although comparable studies have found increased levels of lipid peroxidation products in brains from AD patients, these results are not unequivocal, as several other groups found lipid peroxidation levels to be unchanged (Table 4.5). While earlier studies mostly employed the TBARS (thiobarbituric acid-reactive substances) test for analysis of lipid peroxidation products, this method is prone to produce artefacts arising from interference with further sample components giving false positive results (Janero, 1990). Consequently, recent studies employing more specific and sensitive methodology have produced more consistent results, showing that lipid peroxidation products are mostly increased in AD brains (Lovell et al., 1997; Markesbery and Lovell, 1998; Montine et al., 1998; Nourooz-Zadeh et al., 1999; Karelson et al., 2001; Reich et al., 2001; Musiek et al., 2004). However, comparison of results is complicated by findings that different brain regions exhibit distinct vulnerability or resistance to lipid peroxidation, which is seemingly consistent with a different vulnerability of several brain regions towards the disease progress (Karelson et al., 2001). Additionally, unsaturated lipids and lipid peroxidation products are relatively unstable and prone to undergo decomposition reactions, hence the length of the post mortem interval can affect the outcome of the different studies. Therefore, direct comparisons between different studies are difficult to draw, and conflicting results on lipid peroxidation parameters can be due to a variety of factors including methodology and sample specifications.

DISCUSSION

Table 4.5: Overview on reports of markers for lipid peroxidative damage in AD brains

change observed	parameters studied	brain region	Reference
increased basal and stimulated in FC	TBARS	FC, Cer	(Subbarao et al., 1990)
increased basal and stimulated in FC	TBARS	FC, Hip, Cer	(Richardson, 1993)
increased in FL, motorC, sensC	TBARS	Amyg, Hip, FL, motorC, BNM, PHG, sensC, OC, Cer and others	(Balazs and Leon, 1994)
increased basal and stimulated in ITG	TBARS	ITG, STG, IPL, SFG, sensC, motorC, occipC	(Palmer and Burns, 1994)
increased in Hip, Amyg, Pyr	TBARS	Amyg, Hip, Pyr, MFG, SMTG, IPL, OP, Cer	(Lovell et al., 1995)
increased	HNE	CSF	(Lovell et al., 1997)
increased in Amyg, PHG	HNE	MFG, SMTG, IPL, PHG, Amyg, BNM, Cer	(Markesbery and Lovell, 1998)
increased	F ₂ -isoprostanes	CSF	(Montine et al., 1998; Montine et al., 1999)
increased in PC, TC and Hip	neuroprostanes	PC, TC, Hip, Cer	(Reich et al., 2001)
increased	F ₄ -neuroprostanes	TC	(Musiek et al., 2004)
increased in TC	TBARS	FC, TC, Cer	(Marcus et al., 1998)
increased in FC, OC PC, unchanged in TC	TBARS	FC, OC, PC, TC	(DiCiero et al., 2000)
increased in TC and FC	lipid hydroperoxides	FC, sensPC, OPC, TC	(Karelson et al., 2001)
increased	TBARS	TC	(Yu et al., 2003)
basal unchanged, stimulated increased in PC and TC	TBARS	OC, PC, TC, Cer	(Hajimohammadreza and Brammer, 1990)
unchanged	TBARS	FC	(Hayn et al., 1996)
increased in OL and TL	F ₄ -isoprostanes	OL, PL, TL	(Nourooz-Zadeh et al., 1999)
unchanged	TBARS	FL, PL, OL, TL, STG, MTG, Hip	(Lyras et al., 1997)
basal unchanged, stimulated increased in TC	TBARS	TC, Cer	(McIntosh et al., 1997)
unchanged	TBARS	FC	(Ramassamy et al., 1999)
unchanged	TBARS	Hip	(Ramassamy et al., 2000)

Abbreviations: Amyg = amygdala, BNM = basal nucleus of meynert, Cer = cerebellum, CSF = cerebrospinal fluid, EC = entorhinal cortex, FC = frontal cortex, FL = frontal lobe, FP = frontal pole, Hip = hippocampus, IPL = inferior parietal lobe, ITG = inferior temporal gyrus, motorC = motor cortex, MFG = middle frontal gyrus, MTG = middle temporal gyrus, OC = occipital cortex, OL = occipital lobe, OP = occipital pole, OPC = occipital primary cortex, PC = parietal cortex, PHG = parahippocampal gyrus, PL = parietal lobe, Pyr = pyriform cortex, sensC = sensory cortex, sensPC = sensory postcentral cortex, SFG = superior frontal gyrus, SMTG = superior and middle temporal gyrus, STG = superior temporal gyrus, TBARS = thiobarbituric acid-reactive substances, TC = temporal cortex, TL = temporal lobe.

Studies from cooperating groups, as judged by the author list of the respective publications, are given in the same section.

Due to limitations of measurement of lipid peroxidation products from post mortem material, we additionally analyzed activities of antioxidant enzymes, which are only an indirect measure for oxidative stress-related events, but represent a more stable system as compared to lipid peroxidation products. Analysis of antioxidant enzyme activities revealed that activities of Cu/Zn-SOD, GPx and GR are upregulated in AD patients (Figure 3.34, page 128), which is in good agreement with the majority of other reports (see Table 4.6). Interestingly, these changes in antioxidant enzyme activities were most pronounced in temporal cortex, which is a brain region that is severely affected by AD pathology (Detoledo-Morrell et al., 1997). The higher antioxidant enzyme activities in AD brains may be the result of compensative upregulation following increased oxidative stress, which has been repeatedly shown to occur in cell culture models (Shull et al., 1991; Yoshioka et al., 1994; Tate, Jr. et al., 1995; Jornot and Junod, 1997; Yatin et al., 1998; Yoo et al., 1999; Rohrdanz et al., 2001). Furthermore, significant correlations between antioxidant enzyme activities were found in AD patients, indicating that the whole cascade of antioxidant enzymatic defense is similarly affected (Table 3.6, page 129). This is essentially not surprising as antioxidant defense is afforded by several factors, and the different enzymes act in concert to detoxify ROS.

Our observation that Cu/Zn-SOD enzyme activity was also increased in AD samples from cerebellum was unexpected. Research on cerebellum has been largely neglected in AD research since cerebellar functions are not affected by the disease. Nevertheless, it was found that A β levels are also increased in samples from cerebellum of sporadic AD patients, although the presence of A β containing plaques could not be shown (Larner, 1997). Severe cerebellar A β deposition was only reported in a study of brains from familial AD patients bearing a mutation in PS1 (Lemere et al., 1996). Since A β itself can cause oxidative stress even without being deposited as plaques (Behl et al., 1994b), which we had already observed in studies of PS1 and Thy1-APP transgenic mice, increased cerebellar A β levels may result in higher oxidative pressure on the cells leading to an upregulation of antioxidant enzyme capacity even in the cerebellum. Furthermore, it is possible that oxidative stress is a common feature affecting the whole organism, allowing to detect markers for oxidative stress in several tissues from AD patients, even in peripheral cells [reviewed in (Gibson and Huang, 2002)]. Thus, it can be argued that oxidative markers can similarly be altered in unaffected brain regions like the cerebellum.

DISCUSSION

Table 4.6: Overview of reports on antioxidant enzyme activities in AD brains.

change observed	brain region	Reference
total SOD activity		
increased in NC and CGC	HT, NC, Hip, CGC	(Marklund et al., 1985)
increased in OC	Amyg, Hip, FL, motorC, BNM, PHG, sensC, OC, Cer and others	(Balazs and Leon, 1994)
increased in Hip, Amyg, Pyr, SMT	Amyg, Hip, Pyr, MFG, SMTG, IPL, OP, Cer	(Lovell et al., 1995)
increased in TC	FC, sensC, OPC, TC	(Karelson et al., 2001)
unchanged	FC, PC, TC, OC, basal ganglia, Hip, Amyg, NBM, EC	(Gsell et al., 1995)
unchanged	FC	(Hayn et al., 1996)
unchanged	FC	(Ramassamy et al., 1999)
unchanged	Hip	(Ramassamy et al., 2000)
reduced in FC, Hip, Cer	FC, Hip, Cer	(Richardson, 1993)
reduced in FC, Hip, Cer	FC, Hip, Cer	(Chen et al., 1994)
Cu/Zn-SOD activity		
increased	Hip, SMT, A9	(Bruce et al., 1997)
reduced in FC, TC	FC, TC, Cer	(Marcus et al., 1998)
GPx activity		
increased in Hip	Amyg, Hip, Pyr, MFG, SMTG, IPL, OP, Cer	(Lovell et al., 1995)
increased mRNA	Hip	(Aksenov and Markesbery, 2001)
increased	FC	(Ramassamy et al., 1999)
increased	Hip	(Ramassamy et al., 2000)
unchanged	FC, Hip, Cer	(Richardson, 1993)
unchanged	FC, Hip, Cer	(Chen et al., 1994)
unchanged	FC	(Hayn et al., 1996)
unchanged	FC, TC, Cer	(Marcus et al., 1998)
GR activity		
increased in Hip, Amyg	Amyg, Hip, Pyr, MFG, SMTG, IPL, OP, Cer	(Lovell et al., 1995)
increased mRNA	Hip	(Aksenov and Markesbery, 2001)

Abbreviations: A9 = cortical area 9, Amyg = amygdala, Cer = cerebellum, CGC = cortex gyrus cinguli, EC = entorhinal cortex, FC = frontal cortex, Hip = hippocampus, HT = hypothalamus, NBM = nucleus basalis Meynert, NC = nucleus caudatus, OC = occipital cortex, OPC = occipital primary cortex, PC = parietal cortex, sensC = sensory postcentral cortex, SMT = superior-middle temporal gyrus, TC = temporal cortex.

Studies from cooperating groups, as judged by the author list of the respective publications, are given in the same section.

In summary, our results suggest that cortical areas are effectively protected against lipid peroxidation by the higher antioxidant enzyme activities in AD brains. This is supported by the results of *in vitro* stimulation of lipid peroxidation, where significantly lower

levels of MDA were formed in AD samples (Figure 3.36, page 130). Although we can not exclude that the different formation of lipid peroxidation products after *in vitro* stimulation may be due to a change in lipid composition in AD brains with less amounts of unsaturated lipids that would favour the formation of MDA during the lipid peroxidation process (Soderberg et al., 1991; Prasad et al., 1998), our results confirm previous observations of a higher antioxidant capacity in AD samples (Russell et al., 1999).

4.3.1.2 Gender differences in antioxidant metabolism and oxidative damage in AD patients

Analysis of subgroups of male and female AD patients revealed a gender-specific upregulation of Cu/Zn-SOD and GPx activities especially in female AD patients (Figure 3.38, page 132). No comparable results have been reported so far. In humans, only studies on peripheral tissues are available, reporting higher GPx activity in premenopausal females (Massafra et al., 2002; Rush and Sandiford, 2003), which suggests an inherent gender-specific upregulation of GPx. However, female subjects included in our study were all postmenopausal women older than 60 years. Furthermore, we found no difference between gender in GPx activity in control brains which argues for a rather AD-specific correlation between GPx upregulation and gender than for a general effect of female sex on GPx activity.

Interestingly, GR activities were not increased in female compared to male AD patients. An increase in activities of SOD and GPx without a concomitant increase of GR activity would result in insufficient restoration of reduced glutathione levels. Reduced glutathione is one of the most important cellular antioxidants (Sies, 1999; Schulz et al., 2000) and is critically required for cell survival (Bains and Shaw, 1997). Thus, the lack of GR upregulation in female AD patients would result in higher vulnerability of female brains towards oxidative damage in AD.

Consequently, we found increased levels of HNE specifically in female AD patients compared to male patients (Figure 3.37, page 131). Thus, oxidative damage is present despite the higher activities of SOD and GPx, and the lack of upregulation of GR in brains from female AD patients may lead to oxidative damage. GR activity is required for restoration of reduced glutathione. Apart from being critical to cell survival, reduced glutathione is also a cofactor for the enzyme phospholipid hydroperoxide glutathione peroxidase [reviewed in (Imai and Nakagawa, 2003)], an enzyme that catalyzes the

reduction of lipid hydroperoxides. These hydroperoxides give rise to toxic end products of lipid peroxidation like 4-hydroxynonenal. Furthermore, reduced glutathione is also a cofactor for the detoxification of HNE by glutathione-S-transferases (Spitz et al., 1990; Canuto et al., 1993; Ullrich et al., 1994). Thus, lack of reduced glutathione would result in increased HNE levels as is the case in brains from female AD patients.

A gender difference in the prevalence and incidence of AD has been reported in several studies, with women being at higher risk (Rocca et al., 1986; Jorm et al., 1987; Zhang et al., 1990; Hagnell et al., 1992; Fratiglioni et al., 1997; Seshadri et al., 1997; Gao et al., 1998; Andersen et al., 1999; Copeland et al., 1999; Ruitenberg et al., 2001). Our results show increased activities of antioxidant enzymes in AD brains and especially in brains from female AD patients. On the assumption that higher antioxidant enzyme activities are a compensation for increased oxidative stress, which has been repeatedly shown in cell culture experiments (Shull et al., 1991; Yoshioka et al., 1994; Tate, Jr. et al., 1995; Jornot and Junod, 1997; Yatin et al., 1998; Yoo et al., 1999; Rohrdanz et al., 2001), one might speculate that levels of oxidative stress are higher in brains from female AD patients. Accordingly, levels of HNE were increased in samples from female AD patients. The differences in antioxidant enzyme activities and HNE levels between male and female AD patients might help to explain the above-mentioned epidemiological findings that females have a gender-disparate higher risk to suffer from AD.

This gender-disparate higher risk may be related to higher formation of A β in females (Johnson et al., 1998; Corder et al., 2004) and/or to gender differences in the distribution of plaques (Kraszpulski et al., 2001). Furthermore, this result is in good accordance with our findings on Thy1-APP transgenic mice, where female animals show higher β -amyloidogenic cleavage of APP and oxidative stress earlier than male mice.

It seems possible that gender differences in AD patients are modulated by Apolipoprotein E genotype. Notably, the Apo E4 allele appears to be a risk factor for AD specifically in females (Poirier et al., 1993; Payami et al., 1996; Farrer et al., 1997; Bretsky et al., 1999; Breitner et al., 1999; Molero et al., 2001) and leads to an increased A β plaque load in females (Corder et al., 2004). Additionally, the Apo E4 isoform sensitizes towards A β -induced oxidative stress [see our results on cohort #2 discussed on page 213 and (Ramassamy et al., 1999; Butterfield et al., 2002; Mazur-Kolecka et al., 2002)], and Apo E plays a role in detoxification of HNE, where the Apo E4 allele is least efficient (Pedersen et al., 2000). Taken together, higher vulnerability towards

oxidative stress due to A β in the presence of Apo E4 could be especially pronounced in females. A mechanistic explanation for these observations has been provided by Lambert and colleagues who detected an isoform-specific higher expression of Apo E4 by estrogen (Lambert et al., 2004), although it is questionable whether estrogen effects are of relevance in our study utilizing postmenopausal female patients. Nevertheless, the cohort of samples consisted of a majority of homozygous Apo E4 carriers, with a similar distribution in male (3 homozygous, 1 non-Apo E4) and female (3 homozygous, 3 heterozygous, 1 non-Apo E4) AD patients. Therefore, the increased HNE levels in female AD patients may be the result of a higher vulnerability towards oxidative damage caused by the combined effects of Apo E4 and female gender.

4.3.1.3 Summary of observations in cohort #1

In summary, the results from this study provided evidence that

- (i) activities of antioxidant enzymes are upregulated in AD patients,
- (ii) changes are most pronounced in temporal cortex, which is a severely affected brain region in AD,
- (iii) increased activities of antioxidant enzymes seemingly provide effective protection against lipid peroxidation *in vitro*,
- and
- (iv) antioxidant defense and oxidative damage are modulated by gender, with female AD patients showing a higher vulnerability towards oxidative damage.

4.3.2 Cohort #2

As upregulation of antioxidant enzyme activities was most pronounced in temporal cortex, a larger cohort of samples from temporal cortex was analyzed in a second set of experiments. For these samples, neuropathological and clinical data were available and could be correlated with the results of the biochemical assays.

4.3.2.1 Increased oxidative damage despite partial upregulation of antioxidant defense

In good accordance with the results from cohort #1, basal levels of MDA were not significantly different between controls and AD patients. However, levels of HNE were significantly increased to 132 % of the level in controls (Figure 3.39, page 133). Hence,

HNE seems to be the more specific marker for AD-relevant processes, which is in accordance with a study of lipid peroxidation products in serum from AD patients (McGrath et al., 2001) and also with our results in PS1M146L and Thy1-APP transgenic mice, where only HNE levels, but not MDA levels, were elevated.

Activities of antioxidant enzymes were again increased in AD patients, but significance was only obtained for activities of Cu/Zn-SOD and GPx, whereas the extent of GR upregulation was too small to produce statistical significance (Figure 3.40, page 134). As observed previously, activities of antioxidant enzymes showed highly significant and positive correlations, indicating that the whole cascade of antioxidant enzymes is similarly upregulated, acting in concert to detoxify ROS (Table 3.7, page 134).

The *in vitro* stimulation of lipid peroxidation with ferric iron produced a somewhat unexpected result, as (i) levels of MDA formation were significantly increased in AD samples despite upregulation of antioxidant enzyme activities (Figure 3.42, page 136) and (ii) values were subject to a bimodal distribution that remains unexplained, as none of the available parameters (age, gender, post mortem interval, tissue storage time of samples, activities of antioxidant enzymes, Apo E genotype, disease duration, MMSE score, cause of death or neuropathological Braak staging) could differentiate samples with high levels of MDA formation after stimulation from those with low levels. Thus, either further unknown factors or a combination of several singular factors, which can not be statistically elucidated in our rather small group with 30 subjects, may affect the outcome of MDA formation after *in vitro* stimulation.

4.3.2.2 Correlation of oxidative stress-related parameters with levels of amyloid beta, Apo E genotype and clinical severity of dementia

Correlations with levels of soluble A β_{1-40}

As the amyloid cascade hypothesis centers around the accumulation of A β as the major factor mediating neurotoxicity in AD, we determined levels of soluble A β_{1-40} in a subset of control and AD samples. Although the mean A β_{1-40} levels were greatly increased in AD patients (415 % of controls), this increase was not statistically significant due to a large variation in individual values. Furthermore, some overlap between the control and AD group exists (Figure 3.43, page 138).

A detailed analysis revealed that levels of A β_{1-40} are strongly influenced by the Apo E genotype, with gradually rising levels in the order of E2/E3 < E2/E4 < E3/E3 < E3/E4 <

E4/E4 (Figure 3.44, page 139). This is a perfect reflection of epidemiological studies which found the risk of AD to be lowest in carriers of the E2 allele and highest in E4 carriers (Saunders et al., 1993; Poirier et al., 1993; Corder et al., 1993). The formation of A β plaques in human brains is facilitated by the Apo E4 isoform (Schmechel et al., 1993; Olichney et al., 1996; Berg et al., 1998; Johnson et al., 1998; Ghebremedhin et al., 2001; Tiraboschi et al., 2004a), and a positive correlation between levels of soluble A β_{1-40} and the presence of Apo E4 alleles has been described (Lue et al., 1999). These studies provide evidence that the Apo E4 allele can increase the risk for AD by increasing the formation and/or deposition of A β , thus supporting the amyloid cascade hypothesis. However, as control and AD groups show some overlap, A β_{1-40} levels can not completely differentiate controls from AD patients, suggesting that either A β is not the only factor that provokes the development of AD or that some special form of A β – oligomers, protofibrils or specifically A β_{1-42} – determine the pathogenesis of AD.

Levels of A β_{1-40} showed a positive correlation with MDA levels, which however just failed to reach statistical significance ($p = 0.05$, see Figure 3.45, page 140). A β_{1-40} levels did not correlate with HNE levels or stimulated MDA levels, but were positively correlated with activities of Cu/Zn-SOD and GPx (Figure 3.46 a and b, respectively, page 141), and showed a trend towards positive correlation with GR activity ($p = 0.08$). These results suggest that increased A β_{1-40} levels can promote oxidative damage and at the same time influence the upregulation of activities of Cu/Zn-SOD and GPx, and to a lesser extent also GR. This *in vivo* correlation fits well into *in vitro* observations that A β can induce accumulation of cellular peroxides and upregulate antioxidant enzyme activity (Bruce et al., 1997; Yatin et al., 1998). However, further factors apart from soluble A β_{1-40} may mediate oxidative toxicity in AD brains, as HNE levels, which were significantly increased in AD patients, showed no correlation with A β_{1-40} levels.

Correlations of oxidative stress-related parameters with the Apo E genotype

As stated above, the Apo E4 genotype is a major risk factor for the development of sporadic AD. In order to elucidate a putative causal relationship between the presence of different Apo E isoforms and oxidative stress, the data on lipid peroxidation and antioxidant enzyme activities were evaluated according to the Apo E genotype. Both lipid peroxidation parameters MDA and HNE were lowest in non-E4 carriers (composed of E2/E3 and E3/E3), intermediate in heterozygous E4 carriers (E2/E4 and

E3/E4) and were highest in homozygous E4 carriers (E4/E4) (Figure 3.47, page 142). While differences were not statistically significant for homozygous Apo E4 carriers due to small numbers of subjects in this group ($n = 3$), HNE levels were significantly increased in heterozygous Apo E4 carriers compared to non-E4 carriers. These results are in good accordance with *in vitro* and *in vivo* studies of different Apo E isoforms, where the the Apo E4 isoform was least effective in protecting against oxidative insults (Miyata and Smith, 1996; Butterfield et al., 2002; Mazur-Kolecka et al., 2002), and with studies on human brain tissue, where oxidative damage was related to the number of Apo E4 alleles (Montine et al., 1997; Ramassamy et al., 1999; Ramassamy et al., 2000). The different effects of Apo E isoforms could be due to direct antioxidant properties, as the isoforms differ in two amino acids: while E2 contains two cysteine residues, E3 contains one and E4 contains none. These cysteine residues can either exert direct antioxidant effects through their thiol moiety or bind electrophilic, oxidizing reagents, e.g. lipid peroxidation aldehydes. Thus, Apo E isoforms have been described to differ in their HNE binding ability in the order of $E2 > E3 > E4$ (Pedersen et al., 2000), which can at least partially explain our observations.

Of course, a confounding factor is that the groups of control and AD patients are not homogenously distributed throughout the respective Apo E genotype groups. As stated above, the presence of the Apo E4 allele is a risk factor for the development of AD, while the E2 isoform exerts a protective effect, which is also reflected in our cohort of control and AD samples: the homozygous Apo E4 group consists of only 3 AD patients, the heterozygous group is composed from 3 controls and 6 AD patients, and the non-E4 group represents 6 controls and 5 AD patients. Hence, the Apo E effects in our analysis may not be causally related to the presence of different Apo E isoforms but may instead reflect an inherent elevation of lipid peroxidation products in those groups that are composed of the majority of AD patients, i.e. the heterozygous and above all the homozygous Apo E4 groups. In order to eliminate this confounding fact, we additionally evaluated only the AD and control groups separately. Similar results were obtained in these subgroups, although differences were no longer statistically significant due to smaller numbers of subjects. Of note, although HNE levels increased with rising copies of Apo E4 alleles, levels in the non-E4 and heterozygous Apo E4 AD groups were still higher than in the respective non-E4 and heterozygous Apo E4 control group (Figure 3.49, page 143). Hence it can be concluded that Apo E alleles modulate the extent of HNE formation, but the AD-specific process still leads to a further increase in

HNE levels, which is independent of the Apo E genotype.

An evaluation of putative Apo E allele effects on the activities of antioxidant enzymes produced no statistically significant differences between the different Apo E subgroups. Although enzyme activities were highest in homozygous Apo E4 carriers, this proved to be not statistically significant. While no comparative data on modulation of GR activity by Apo E genotype are available, total SOD activity was reported to be unchanged with Apo E4 genotype in frontal cortex as well as hippocampus (Ramassamy et al., 1999; Ramassamy et al., 2000). Reports on GPx activity are conflicting and seem to depend on the brain region analyzed: GPx activity in homozygous E4 AD patients compared to homozygous E3 patients has been reported to be increased in frontal cortex (Ramassamy et al., 1999) but decreased in tissue from hippocampus (Ramassamy et al., 2000). No comparable data are available for temporal cortex. Hence the overall conclusion from our results would be that the Apo E genotype does modify the extent of oxidative damage, but does not have a major impact on the activities of antioxidant enzymes.

Correlations of oxidative stress-related parameters with mini mental status MMSE

Among the set of samples, the clinical degree of cognitive impairment as assessed by the mini mental status examination MMSE was available for 11 AD patients and 2 controls. The controls scored 28 and 29 points, respectively, which is within the expected values for cognitively normal subjects [usually, normal values are ranging from 24 points to 30 points, depending on educational level and age (Osterweil et al., 1994; Ostrosky-Solis et al., 2000)]. The AD patients scored a mean value of 8.6 points, with individual values ranging from 0 to 21 points.

Correlation of lipid peroxidation products with MMSE scores in individual samples produced divergent results for MDA and HNE levels. MDA levels were negatively correlated with MMSE scores ($p = 0.055$, Figure 3.50, page 144), indicating that MDA formation accompanies the progression of the disease. Whether increased MDA levels do significantly contribute to the progression of AD or are just a consequence of increased neurodegeneration remains to be elucidated. Levels of HNE however show a different pattern with disease progression: while HNE levels are high in AD patients with moderate cognitive impairment, they decrease in subjects with severe cognitive impairment (Figure 3.51, page 145). This suggests that HNE levels are highest in earlier stages of the disease and recede to the almost levels of controls when the disease is

proceeding to a severe state. Interestingly, similar observations have been made in a study by Nunomura and coworkers, who reported that immunohistochemically detected levels of nitrotyrosine residues and 8-hydroxyguanine as markers for oxidative stress were quantitatively greatest in early clinical stages of the disease and reduced with disease progression (Nunomura et al., 2001). These findings led the authors to voice the provocative statement that “oxidative damage is the earliest event in AD”. Although our results do not unambiguously support this, as MDA levels increase with disease progression, it is nevertheless possible that different markers for oxidative damage detect different processes during clinical progression of AD and hence can be distinctly changing with increasing severity of the disease. As HNE levels are especially high in earlier clinical stages of the disease, neurotoxicity due to HNE may play an important role in the initiation of early AD-related neurodegenerative events.

In this respect, it is interesting to analyze a putative time-course of neuropathologic changes in AD. It seems that early intraneuronal accumulation of A β is one of the primary events, whereas extracellular A β plaques are formed at later stages after neuron death (LaFerla et al., 1997; Gouras et al., 2000). Tau pathology seems to be initiated after A β accumulation and to increase constantly during disease progression, as do gliosis and synaptic loss (Rozemuller et al., 1989; Naslund et al., 2000; Ingelsson et al., 2004; Schonheit et al., 2004; Tiraboschi et al., 2004b). Progressive gliosis caused by amyloid deposition may lead to inflammatory reactions accompanied by additional ROS formation by activated microglia and astrocytes in AD brains (McGeer and McGeer, 1995; Meda et al., 1995; Klegeris and McGeer, 1997; McDonald et al., 1997; Bianca et al., 1999; Wang et al., 2002). Considering such a time-course of events, it may be possible that increased HNE levels in earlier stages of the disease reflect initial oxidative stress caused by accumulation of intraneuronal A β . As intraneuronal A β disappears at later stages, HNE levels simultaneously recede, which would be consistent with our observations in Thy1-APP transgenic mice, where only HNE levels, but not MDA, were elevated in young female and middle-aged mice, but HNE levels later returned to the levels of non-transgenic mice in 18 months old animals.

Contrary to HNE, the constantly rising MDA levels during clinical progression of AD may represent oxidative damage related to inflammatory reactions and progressive gliosis. A putative mechanism for MDA formation under conditions of inflammation may be represented by cyclooxygenase reactions, where the pentacyclic ring bridged by an epidioxy moiety can decompose under formation of MDA (Spiteller, 2001). Both

increased expression of cyclooxygenase 1 and 2 has been identified in AD brains (Kitamura et al., 1999). Hence, different neuropathological processes related to intraneuronal A β accumulation or inflammatory reactions triggered after extracellular deposition of A β may be accompanied by distinct changes in lipid peroxidation parameters. Exploration of the mechanisms that lead to formation of the different lipid peroxidation aldehydes *in vivo* may shed more light on this issue.

Analysis of antioxidant enzyme activities suggests that different antioxidant responses are activated during the progression of AD. Both Cu/Zn-SOD and GPx activity showed highest activity in the moderately affected AD patient group (Figure 3.52, page 146). While Cu/Zn-SOD activity showed a trend towards higher activity, GPx activity was significantly increased in this group. Both enzyme activities however receded towards lower levels in the severely impaired AD patient group, although they were still higher than values of controls, but not statistically significant. In contrast to Cu/Zn-SOD and GPx activity, GR activity shows no such peak in the moderately affected AD patient group. Instead, GR activity was highest in the group of severely cognitively impaired AD patients, although not statistically significant (Figure 3.52, page 146). Hence, Cu/Zn-SOD and GPx activities seem to be the enzymes that respond early to oxidative pressure, whereas upregulation of GR activity is a rather late event. Comparing the dynamic alterations in antioxidant enzyme activities with those of HNE levels, it can be speculated whether the lack of GR upregulation at early stages of the disease is responsible for the observed increase in HNE levels. Insufficient restoration of reduced glutathione GSH by GR may lead to accumulation of HNE, as glutathione-dependent detoxification of HNE by glutathione-S-transferase as well as reduction of lipid hydroperoxides by PH-GPx would be compromised. The selective contribution of different antioxidant enzymes to the accumulation of lipid peroxidation aldehydes should be explored in future studies. This may provide some more insight into the question *if* and, more importantly, *how* different antioxidant enzyme activities can specifically influence the formation of MDA or HNE.

In summary, although the results should be considered preliminary, as the statistical power with only 11 AD subjects is limited, they provide evidence that dynamic changes of several oxidative stress-related parameters can take place during the progression of AD. Whether the changing pattern of antioxidant enzyme activities during the progression of AD does significantly contribute to the accumulation of neurotoxic HNE, and whether this has impact on the initiation and progression of sporadic AD will pose a

challenging field of research for future studies. Rigorous analysis of these changes and identification of pharmacological substances that allow for a selective modification of these pathologic events may offer an approach for development of therapeutic agents that can ameliorate disease progression once it has started.

4.3.2.3 Summary of observations in cohort #2

In summary, the results from this study provided evidence that

- (i) activities of antioxidant enzymes Cu/Zn-SOD and GPx, but not GR, are upregulated in AD patients,
 - (ii) basal levels of HNE, but not MDA, are increased in AD patients,
 - (iii) the increase in activities of Cu/Zn-SOD and GPx does not provide protection against stimulation of MDA formation *in vitro*,
 - (iv) accumulation of A β , which is strongly modulated by the Apo E genotype, can promote oxidative damage and upregulation of antioxidant defenses, since A β levels are positively correlated with MDA levels and activities of Cu/Zn-SOD and GPx,
 - (v) the presence of Apo E4 alleles increases oxidative damage without an impact on antioxidant enzymes,
- and
- (vi) dynamic changes occur during the progression of the disease: while HNE levels are highest in earlier stages, MDA levels are steadily increasing with clinical progression of dementia severity.

4.3.3 Comparison of results from cohort #1 and #2

Summarizing the changes in oxidative stress-related parameters observed in the two different cohorts of controls and AD patients, some parameters produced conflicting results while others could be independently reproduced in the second set of samples. Table 4.7 summarizes the main outcomes of both studies, considering only the data from temporal cortex in cohort #1.

The differences between the two cohorts are a good reflection of the various conflicting findings in comparable reports, as mentioned earlier (see Table 4.5 for lipid peroxidation products and Table 4.6 for antioxidant enzymes). Therefore, the results of a single study should not be overemphasized, especially as the evaluation of cohort #2

provided evidence that sample properties like Apo E genotype, clinical progression of the disease and A β levels can modulate oxidative stress-related parameters. Hence, further studies utilizing still larger sets of samples may prove beneficial in elucidating multifactorial relationships between oxidative stress markers and sample properties.

Nevertheless, it can be concluded from Table 4.7 that measurement of Cu/Zn-SOD and GPx activity produces the most reliable results, as an upregulation of these enzyme activities in AD patients relative to controls was found in both independent studies. However, the *extent* of upregulation expressed as percentage relative to controls was different in the two studies: in cohort #1 the differences were in general more pronounced than in cohort #2 (Table 4.8).

Table 4.7: Comparison of results from two independent studies on human brain tissue samples.

Parameter	cohort #1	cohort #2
basal MDA levels	o	o
basal HNE levels	o	+
stimulated MDA levels	o ¹	+
Cu/Zn-SOD activity	+	+
GPx activity	+	+
GR activity	+	o
gender difference female / male AD patients in Cu/Zn-SOD, GPx or HNE	o ¹	o

+ indicates significant increase, o indicates no significant change relative to controls.

¹ differences in cohort #1 were only significant in Twoway-ANOVA over the 4 brain regions analyzed, but not in temporal cortex alone.

Table 4.8: Relative increases in activities of antioxidant enzymes in AD brain tissue from temporal cortex compared to non-demented controls in cohort #1 and #2.

Study	Enzyme	Cu/Zn-SOD	GPx	GR
Cohort #1	relative increase (%)	23.4	41.1	16.8
	t-test	p < 0.01	p < 0.01	p < 0.05
Cohort #2	relative increase (%)	19.2	20.9	6.4
	t-test	p < 0.05	p < 0.05	n.s.

The differences in upregulation of antioxidant enzyme activities may help to explain some of the discrepancies between the two studies. Thus, the lack of an increase in basal HNE levels in AD patients seen in Cohort #1 might be related to the finding that relative increases in antioxidant enzymes are higher in this cohort, providing a higher degree of protection against oxidative damage. Furthermore, GR activity was not significantly increased in cohort #2. Hence insufficient GR upregulation might promote oxidative damage at the level of HNE. Similar observations were made

(i) in brains from female AD patients in cohort #1, where only Cu/Zn-SOD and GPx activity, but not GR activity, were significantly increased, which was accompanied by increased levels of HNE,

and

(ii) in the correlation of oxidative stress-related parameters with the clinical degree of cognitive impairment, where HNE levels were highest accompanied by increased Cu/Zn-SOD and GPx, but not GR activity, in the group of AD patients with moderate cognitive impairment.

Hence, insufficient upregulation of GR activity may specifically result in increased HNE accumulation. GR activity is needed for restoration of reduced glutathione in the cell, which is also a cofactor for the enzyme phospholipid hydroperoxide glutathione peroxidase [reviewed in (Imai and Nakagawa, 2003)] that catalyzes the reduction of lipid hydroperoxides. These hydroperoxides are precursors of toxic lipid peroxidation end products like HNE. Furthermore, reduced glutathione is a cofactor for the detoxification of HNE by glutathione-S-transferases (Spitz et al., 1990; Canuto et al., 1993; Ullrich et al., 1994). Hence, an insufficient restoration of reduced glutathione by the GR reaction may lead to accumulation of HNE.

Also, the extent of MDA formation after *in vitro* stimulation with ferric iron may be related to the activities of antioxidant enzymes: in cohort #1 enzyme activities were highly upregulated resulting in efficient inhibition of lipid peroxidation in all brain regions, whereas the limited upregulation of enzyme activities does not afford protection in cohort #2.

Another major difference in our results between the two studies is the lack of a gender difference in upregulation of antioxidant enzyme activities and HNE levels in cohort #2. It must be noted that gender differences in cohort #1 were only significant in Two-way ANOVA over all brain regions but not statistically significant in temporal cortex alone.

Nevertheless the larger number of subjects employed in cohort #2 could have overcome the weak statistical significance. The mean age at death was not different between AD patients from cohort #1 and #2, also the major cause of death was similar between the two cohorts, where most AD patients died of pulmonary insufficiency. However, in cohort #2, we found significant effects of MMSE, a measure of cognitive decline, on HNE levels and GPx activity as well as a tendency on affecting Cu/Zn-SOD activity. Hence the clinical state of disease progression could have affected the results of gender differences in cohort #1. Unfortunately, no clinical data were available for patients in cohort #1. Therefore, it can only be speculated which factors could have influenced the different outcome.

A likely explanation for the conflicting results on gender differences in oxidative stress-related parameters in AD patients could nevertheless reside in the strikingly different distribution of Apo E alleles between the two cohorts: in cohort #1 the majority of AD patients were homozygous Apo E4 carriers, whereas in cohort #2 only a minority of patients was homozygous for Apo E4 (see Table 4.9).

Table 4.9: Comparison of Apo E genotype of AD patients in cohort #1 and #2.

Set of samples	unknown	non-Apo E4	hetero-zygous E4	homo-zygous E4
Cohort #1				
Total AD patients		2	2	5
female		1	2	3
male		1	0	2
Cohort #2				
Total AD patients	2	5	6	3
female	2	2	3	1
male	0	3	3	2

Hence, the relative numbers of Apo E4 homozygous subjects in cohort #1 (5 of 9 AD patients) are higher than in cohort #2 (3 of 14 AD patients, 2 patients with unknown Apo E genotype). As the Apo E4 genotype was shown to be associated with a higher risk for AD especially in females (Poirier et al., 1993; Payami et al., 1996; Farrer et al., 1997; Bretsky et al., 1999; Breitner et al., 1999; Molero et al., 2001) and numerous interactions of Apo E and estrogens have been described [reviewed in (MacLusky, 2004)], the relatively higher numbers of Apo E4 carriers in cohort #1 could have produced the gender difference in HNE levels and upregulation of antioxidant enzymes Cu/Zn-SOD and GPx, while no such gender difference was observed in cohort #2. However, the numbers of subjects in both cohorts are too small to reevaluate the data

with respect to gender-disparate changes in oxidative stress-parameters in only the subgroup of Apo E4 homozygous AD patients. Therefore, this issue can only be investigated in more detail in a still larger cohort of samples.

A further difference between cohort #1 and cohort #2 is present in the mean post mortem interval, with a mean PMI (mean \pm 95 % C.I.) of 24.52 ± 4.36 hours in samples from cohort #1 and only 5.0 ± 0.84 hours in cohort #2. This can explain the differences in the absolute levels of lipid peroxidation products between the two cohorts. Oxidized lipids are chemically instable and probably prone to undergo decomposition reactions during the PMI interval, resulting in the observed higher basal levels of lipid peroxidation products in cohort #1 as compared with cohort #2, whereas the stimulation of MDA formation was less pronounced in cohort #1 – probably due to already substantial decay of lipids in tissue samples during the longer PMI, resulting in less efficient *in vitro* stimulation and consequently lower levels of MDA formation in cohort #1 as compared to cohort #2.

Nevertheless, the major finding that activities of antioxidant enzymes are specifically upregulated in AD patients could be independently reproduced in cohort #2. This may be related to the fact that activities of antioxidant enzymes obviously remain relatively stable during the post mortem interval (Brannan et al., 1982). Hence the outcome of the results was similar between the two cohorts despite significant differences in post mortem interval. Of course, it can be speculated which factors may mediate the upregulation of antioxidant enzymes. Cell culture models have shown that treatment with oxidative stressors leads to an increased expression and/or activity of several antioxidant enzymes (Shull et al., 1991; Yoshioka et al., 1994; Tate, Jr. et al., 1995; Jornot and Junod, 1997; Yatin et al., 1998; Yoo et al., 1999; Rohrdanz et al., 2001), hence the upregulation of antioxidant enzymes may be a response to increased ROS accumulation in AD brains. In accordance with this, increased levels of HNE as a marker for oxidative damage were found in AD brain in cohort #2, suggesting that AD brains operate under increased oxidative pressure. However, the exact factors which mediate the upregulation of antioxidant enzymes on a molecular level have yet to be identified.

So far, several putative binding sites have been identified in the upstream region of the human Cu/Zn-SOD gene (Kim et al., 1994), among these sites for NF- κ B and Ap-1 binding. NF- κ B and Ap-1 have also been shown to be important for GPx and catalase

transcription in response of muscle cells to oxidative stress (Zhou et al., 2001). Regulation of GR transcription in mammalian cells is unknown, but dependence on Ap-1 has been described in yeast (Grant et al., 1996). Thus, NF- κ B and Ap-1 are the most widely studied factors in mediating transcriptional regulation of a variety of antioxidant enzymes, although other factors probably play a role as well (Seo et al., 1996; Merante et al., 2002; Ermak et al., 2004; Hussain et al., 2004).

In this respect, A β and/or APP-derived proteins have been shown to activate a variety of stress responses *in vitro* – including activation of NF- κ B (Behl et al., 1994b; Barger and Mattson, 1996; Akama et al., 1998; Cardoso and Oliveira, 2003; Casal et al., 2004) and the MAP kinase pathway (Greenberg et al., 1994; Anderson et al., 1995; Tamagno et al., 2003; Marques et al., 2003) with subsequent c-jun kinase activation that could influence AP-1 DNA binding activity (see 1.1.3.4, page 13). NF- κ B and c-jun kinase activation could also be detected in brains from AD patients (Anderson et al., 1994; Kaltschmidt et al., 1997; Zhu et al., 2001). Furthermore, A β has been shown to induce Cu/Zn-SOD activity *in vitro* (Bruce et al., 1997; Yatin et al., 1998; Cardoso and Oliveira, 2003).

Apart from A β , also HNE – the lipid peroxidation aldehyde that was specifically elevated in AD patients and moreover elevated at early stages of the disease (see Figure 3.39, page 133, and Figure 3.51, page 145) – has been shown to activate MAP kinases (Tamagno et al., 2003), AP-1 binding (Camandola et al., 2000a) and to induce the heat shock transcription factor (Cajone and Crescente, 1992). Interestingly, in rat astroglial cultures, HNE treatment modified GPx and GR activity in a way similar to our observations, resulting in increased GPx but decreased GR activity (Ahmed et al., 2002). This resembles the situation in human AD brains in cohort #2 with moderate MMSE scores, where HNE levels are elevated accompanied by increased GPx, but not GR activity (see Figure 3.51, page 145, and Figure 3.52, page 146).

In conclusion, A β accumulation in AD brains could – in concert with other factors that have yet to be identified – result in increased oxidative stress, accumulation of HNE and a direct or indirect activation of protective antioxidant cascades. Furthermore AICD, the intracellular domain of APP resulting from γ -secretase cleavage, can act as a transcription factor (Leissring et al., 2002), but target genes are yet unidentified. Hence, several putative mechanisms of gene activation can be induced by APP and APP-derived proteins or A β , either directly or indirectly, e.g. via increased ROS production

and/or HNE formation. These mechanisms may explain why activities of antioxidant enzymes are upregulated in AD patients in our studies. However, upregulation of antioxidant enzymes is either not sufficient to protect against accumulation of oxidative damage, or the relative failure in upregulation of GR activity may result in insufficient protection against accumulation of HNE.

Summary of observations in human AD brain tissue

Figure 4.6 summarizes the results obtained in studies of human AD brain tissue in the course of this thesis.

Taken together, our results from two independent studies of human brain tissue show that activities of Cu/Zn-SOD and GPx and to a lesser extent GR are elevated in AD patients relative to non-demented controls. This upregulation of antioxidant enzyme activities probably occurs in response to increased ROS levels but is not sufficient to protect against oxidative damage, because elevated levels of the lipid peroxidation product HNE were detected in AD brains.

Furthermore, alterations in oxidative stress-related parameters were subject to modulation by important sporadic AD risk factors. Accordingly, activities of Cu/Zn-SOD and GPx are even more profoundly increased in female AD patients accompanied by increased HNE levels, suggesting that female gender predisposes to pathogenic alterations in oxidative stress-related parameters, which is consistent with epidemiological reports of a higher risk for sporadic AD in women. Moreover, the Apo E genotype has an impact on the extent of oxidative damage, as MDA and HNE levels were dose-dependently increased in Apo E4 carriers. Additionally, levels of A β were correlated with MDA levels and activities of Cu/Zn-SOD, GPx and to a lesser extent GR, suggesting that soluble A β can be an important mediator of oxidative damage and antioxidant response in brains from AD patients.

Finally, dynamic alterations in oxidative stress-related parameters take place during the progression of the disease: while HNE levels are highest in moderately cognitively impaired AD patients, MDA levels are steadily increasing with clinical progression of the disease. Hence, HNE may be a relevant neurotoxic factor in earlier disease stages whereas MDA can constitute a continuously rising damaging factor during the disease progression.

In conclusion, the changes in oxidative stress-related parameters identified in brains

from sporadic AD patients, the modulation by important AD risk factors like female gender and the Apo E4 genotype as well as the correlation with levels of soluble A β and the clinical status of dementia support the hypothesis that oxidative stress plays a major role in AD-relevant pathogenic processes. The identification of the molecular links between A β accumulation, APP processing and upregulation of antioxidant enzyme activities can therefore provide new pharmacological targets for augmentation of antioxidant defenses in AD brains.

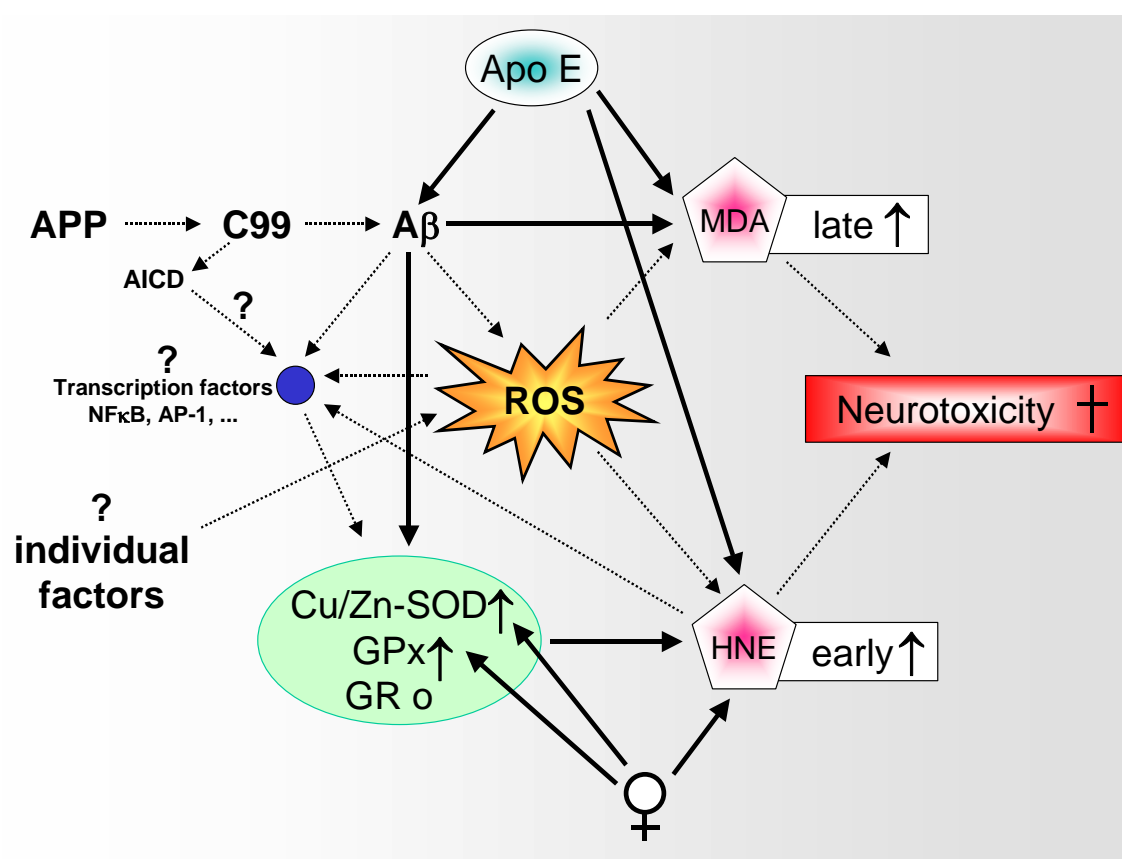


Figure 4.6: Summary of effects on oxidative stress-related parameters observed in brain tissue from sporadic AD patients.

Accumulation of A β in AD brains, which is strongly modified by the Apo E genotype, leads to an upregulation of antioxidant enzymes, mostly Cu/Zn-SOD and GPx. GR activity is however not sufficiently upregulated, which may facilitate accumulation of toxic HNE. Activities of antioxidant enzymes Cu/Zn-SOD and GPx and HNE levels as a measure of oxidative damage are furthermore modified by gender. While HNE is elevated early in the disease progression, MDA is highest at later stages and correlates with the levels of A β . Levels of MDA and HNE are modulated by the Apo E genotype. See text for further details.

Straight lines represent findings from this thesis, whereas dotted lines represent well-established events from other reports. Hypothetical events are marked by questionnaires.

4.4 Comparative summary and perspectives

The results obtained in the course of this thesis allow for an estimation of the contribution of oxidative stress to the pathogenesis of AD and for a comparison of relevant transgenic mouse models with the situation in brains from sporadic AD patients. The suitability of transgenic mice as models for AD, the common findings in mice and sporadic AD patients that HNE but not MDA levels are selectively increased and that oxidative stress-related parameters are subject to modification by gender will be discussed here in more detail. Furthermore, the results of this thesis will be reviewed with respect to strategies utilizing antioxidants for the prevention and/or therapy of AD.

4.4.1 Suitability of transgenic mice to study AD-relevant pathogenic mechanisms

The generation of different lines of transgenic mice expressing human proteins with familial AD mutations has provided valuable models for AD research [reviewed in (Seabrook and Rosahl, 1999; Hock, Jr. and Lamb, 2001; Higgins and Jacobsen, 2003)]. One of the most important steps in mimicking AD pathology in mouse brains was the generation of APP transgenic mouse models, which display accumulation of A β and formation of plaques at later stages (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997) and reactive gliosis (Frautschy et al., 1998; Stalder et al., 1999). Furthermore, neuronal loss has been described in some models (Calhoun et al., 1998; Schmitz et al., 2004), although some of these results are controversial (Irizarry et al., 1997; Takeuchi et al., 2000), probably due to methodological difficulties inherent to assessment of neuron counts (Dickson, 2004).

Despite the progress in modelling AD pathology in transgenic mice, it can not be denied that these mouse models have some limitations. Confounding factors that have been observed in several APP transgenic mice are behavioural abnormalities like hyperactivity and/or aggressiveness (Moechars et al., 1996; Moechars et al., 1998; Van Dam et al., 2003), a phenotype which was also present in Thy1-APP and APP23 transgenic mice employed in this thesis. Although the occurrence of neuropsychiatric abnormalities like increased irritability and aggressive behaviour has been documented in a large percentage of AD patients (Devanand, 1997; Hope et al., 1999), it is not clear whether the behavioural disturbances in mice are causally linked to AD-relevant processes due to APP/A β expression or whether they constitute a confounding artefact.

The fact that relatively high levels of overexpression of APP are needed to elicit the deposition of A β plaques raises concerns about possible artefacts due to unphysiological protein levels. Knowledge of further factors that can influence A β deposition in mice may lead to development of mouse models with more physiological protein levels. In this respect, several studies provided evidence that factors related to immune activation can modulate accumulation of A β (Wyss-Coray et al., 1997; Wyss-Coray et al., 2001; Little et al., 2004). Furthermore, disruption of estrogen receptor beta results in A β deposits in non-transgenic mouse brains (Zhang et al., 2004), and environmental enrichment accelerates plaque deposition in APP transgenic mice (Jankowsky et al., 2003). Thus, augmentation of plaque formation by similar approaches in APP-bearing mice may allow for the development of AD mouse models without unphysiological levels of APP overexpression.

Furthermore, the plaques formed in transgenic mouse brains during the relatively short life span of 2-3 years are distinctly different from plaques in human brains accumulating over a much longer period of time (Kuo et al., 2001; Kalback et al., 2002). In addition, APP transgenic mice mimic plaque pathology to some extent, but not the extensive neurodegeneration and tau pathology as observed in human brains. The recent development of triple transgenic mice expressing mutant APP, PS1 and tau protein (Oddo et al., 2003) may overcome some of these problems. Importantly, neurofibrillary pathology in mice bearing mutant tau protein depends on the presence of A β (Götz et al., 2001; Lewis et al., 2001), giving further support to the amyloid hypothesis.

Finally – despite progress in the development of mice with plaque *and* neurofibrillary tangle pathology – transgenic mice do not mimic the whole pathology seen in AD brains. For example, microglial activation and immune responses in APP transgenic mice are different from the situation in human AD brains (Schwab et al., 2004), which may explain why our results on several transgenic mouse lines are different from the situation in human AD brains (see section 4.3). Although oxidative stress could be detected in PS1 and Thy1-APP transgenic mice, no such observation was made in PDGF-APP or PDGF-APP/PS1 mice. Hence, the deposition of plaques is not inevitably associated with increased oxidative stress. Similarly, an impairment of Cu/Zn-SOD activity was only found in Thy1-APP and APP23 transgenic mice, but not in the other transgenic mouse models, whereas activities of antioxidant enzymes were clearly upregulated in sporadic AD patients. Hence it can be concluded that oxidative damage as elevated HNE levels in sporadic AD brains can be reproduced in some but not all

transgenic mouse models, whereas the upregulation of antioxidant enzyme activities seen in AD brains is completely absent in the transgenic mice analyzed in this thesis. It seems that especially adaptive processes towards oxidative damage are differently regulated in transgenic mouse brains compared to human brains. To close the gap between mouse models based on FAD mutations and sporadic AD patients, it would be necessary to analyze brain tissue from FAD cases, which are however very rare and therefore difficult to obtain. Such studies would allow to elucidate (i) whether the pathogenesis of both familial and sporadic AD results in the same final pathway, i.e. neurotoxicity mediated by oxidative stress and increased HNE levels, and also (ii) whether this final pathway is caused by different mechanisms, i.e. impaired antioxidant defense in carriers of APP mutations and increased ROS production by PS1 mutations in contrast to insufficient upregulation of antioxidant enzymes in sporadic AD patients.

Furthermore, our results on APP transgenic mice allow to conclude that oxidative stress is not a feature of amyloid deposition under all conditions, as we detected neither increased HNE nor MDA levels in brains from PDGF-APP/PS1 transgenic mice at any age, despite plaque formation from an age of 6 months (Blanchard et al., 2003). In this respect it has to be noted that the amyloid hypothesis in its original form had to be modified in recent years to better fit all experimental evidence (Hardy and Selkoe, 2002). Reports that A β is neurotrophic (Whitson et al., 1989; Whitson et al., 1990; Yankner et al., 1990) and is formed also under physiological conditions (Haass et al., 1993) have stimulated the search for a special toxic species of A β . Accumulating evidence has been provided that A β plaques are probably not a major factor initiating pathogenic processes but can be rather considered as a non-toxic epiphenomenon caused by misguided APP and/or A β processing. Several factors argue for this opinion: neurodegeneration has been observed in PS1 mice without formation of amyloid plaques (Chui et al., 1999), cognitive deficits and elevated oxidative stress both precede the deposition of plaques (Pratico et al., 2001; Van Dam et al., 2003) and neurodegeneration exceeds the areas of plaque deposition (Schmitz et al., 2004). Furthermore, the plaque load in humans gives only a poor correlation with the clinical status of cognitive impairment in human AD patients (Braak and Braak, 1991; Nagy et al., 1995; Gomez-Isla et al., 1997; Giannakopoulos et al., 2003; Guillozet et al., 2003). Instead, soluble A β species, i.e. oligomers up to 100 kDa, or protofibrils (Kuo et al., 1996; Walsh et al., 1999; Haass and Steiner, 2001; Klein et al., 2001; Takahashi et al., 2002; Gong et al., 2003) may constitute the main neurotoxic agents. The rigorous analysis of these A β

species in human and transgenic mouse brains may provide better ways of identifying mouse models which more closely mimic AD-relevant pathogenic events.

4.4.2 Is HNE a major toxic factor in AD?

It is striking that we found specifically elevated levels of HNE, but not MDA, in brains from sporadic AD patients (cohort #2) as well as in two different mouse models based on familial AD mutations: in aged mice transgenic for mutant PS1 and in young and middle-aged Thy1-APP transgenic mice bearing the Swedish and London mutations in APP. Hence it can be speculated whether increased HNE levels are a common toxic principle in sporadic as well as familial AD.

First of all, a large body of evidence points to an increase in HNE levels in brain and CSF from sporadic AD patients (Lovell et al., 1997; Markesbery and Lovell, 1998), which is supported by studies of HNE-modified proteins (Sayre et al., 1997; Ando et al., 1998; Butterfield et al., 2002). Furthermore, increased HNE but not MDA levels were found in serum from AD patients compared to controls (McGrath et al., 2001), which confirms our observations that selectively HNE levels are increased in brains from AD patients and transgenic mouse models.

HNE has been shown to be formed *in vitro* after treatment of cell cultures with A β (Mark et al., 1997; Tamagno et al., 2003), and HNE displays neurotoxic properties in several models (Kruman et al., 1997; Soh et al., 2000; Malecki et al., 2000; Camandola et al., 2000a). Neurotoxic mechanisms described for HNE include activation of c-jun kinase (Soh et al., 2000; Tamagno et al., 2003) and mitochondrial toxicity – ranging from inactivation of mitochondrial proteins like cytochrome c oxidase (Chen et al., 1998; Chen et al., 1999), complex II and III of the respiratory chain (Picklo et al., 1999), the adenine-nucleotide translocator (Vieira et al., 2001), α -ketoglutarate dehydrogenase (Humphries and Szweda, 1998; Korotchkina et al., 2001) and pyruvate dehydrogenase (Korotchkina et al., 2001) to an alteration of mitochondrial membrane fluidity (Chen and Yu, 1994). Of note, deficiencies in cytochrome c oxidase (Wong-Riley et al., 1997; Maurer et al., 2000; Bosetti et al., 2002) and the pyruvate dehydrogenase complex activity (Sorbi et al., 1983) have been identified in brains from AD patients. Furthermore, HNE can interfere with neuronal signaling by an impairment of intracellular calcium homeostasis via inactivation of ion-motive ATPases (Mark et al., 1997; Siems et al., 2003), by interaction with G proteins (Blanc et al., 1997) and protein kinase C (Chiarpotto et al., 1999), and by inactivation of glutamate transport into

glial cells (Blanc et al., 1998; Lauderback et al., 2001). Finally, HNE inhibits dephosphorylation of tau (Mattson et al., 1997) and induces proteasomal dysfunction (Hyun et al., 2002), which can propagate the development of abnormal protein accumulation and NFT pathology in AD brains. Hence, several mechanisms of impaired neuronal function, neurotoxicity and progression of AD neuropathology can be mediated by HNE.

A major question to be answered is why HNE, but not MDA levels, are increased in mutant PS1 and Thy1-APP transgenic mice as well as in AD patients. Mechanisms of formation of these aldehydes *in vivo* are difficult to elucidate. *In vitro* studies of lipid peroxidation systems revealed that the structural requirement for HNE formation are present in lipid hydroperoxides from n-6 polyunsaturated fatty acids (Pryor and Porter, 1990; Schneider et al., 2001a) – which can arise from cyclooxygenase or lipoxygenase reactions with arachidonic acid. Interestingly, increased immunostaining for 12/15-lipoxygenase has been identified in brains from AD patients (Pratico et al., 2004). The reaction of 12/15-lipoxygenase with e.g. arachidonic acid can provide lipid hydroperoxides that have the structural prerequisite for formation of HNE. In contrast, MDA can be formed from a larger variety of unsaturated fatty acids due to less structural requirements, which may explain why MDA is the more abundant lipid peroxidation product. n-6 polyunsaturated fatty acids like arachidonic acid yield high levels of HNE after microsomal oxidation (Esterbauer et al., 1986) or oxidation with iron ascorbate (Mlakar and Spiteller, 1996). Recently it was also shown that HNE can be formed *in vitro* from oxidation of lipid hydroperoxides by heme-iron-containing proteins like cytochrome c (Hayashi et al., 2004), which is present in complex IV of the mitochondrial respiratory chain. Hence, disturbed mitochondrial functions, loss of mitochondrial membrane potential, increased mitochondrial ROS formation and release of cytochrome c from the respiratory chain may favour the formation of HNE, especially as mitochondrial membranes have a high content of PUFAs (Zabelinskii et al., 1999; Gutierrez et al., 2002).

Previous studies from our own group and others have linked APP overexpression and/or exposure to A β with mitochondrial damage (Canevari et al., 1999; Casley et al., 2002; Aliev et al., 2003; Marques et al., 2003), which was also detected in Thy1-APP transgenic mice (Blanchard et al., 2003; Keil et al., 2004). Furthermore, HNE levels in brain tissue and mitochondrial ROS levels in peripheral cells were positively correlated in the study on PS1 transgenic mice, supporting the notion that HNE can arise from

mitochondrial ROS. Therefore, a hypothetical mechanism of formation of HNE *in vivo* after mitochondrial damage, arising specifically from lipid hydroperoxide formation by mitochondrial ROS and from further oxidation by cytochrome c can be postulated. Alternatively, HNE could be the mediator of the toxic effects of A β on mitochondria (Keller et al., 1999). Both mechanisms should be explored in future studies.

4.4.3 Gender differences in AD and hormone replacement therapy

A gender difference in the prevalence and incidence of AD has been widely described, with women being at higher risk, even when the longer life expectancy for women is taken into account and data are compared to age-matched men (Rocca et al., 1986; Jorm et al., 1987; Zhang et al., 1990; Hagnell et al., 1992; Fratiglioni et al., 1997; Seshadri et al., 1997; Gao et al., 1998; Andersen et al., 1999; Copeland et al., 1999; Ruitenberg et al., 2001). Although these findings are still controversial (Rocca et al., 1998; Yamada et al., 1999; Ganguli et al., 2000; Hebert et al., 2001; Graff-Radford et al., 2002; Fitzpatrick et al., 2004) and may be confounded by a longer survival after diagnosis of AD in women (Corder et al., 1995), the gender difference is still striking. Furthermore, a gender-specific higher risk for AD in women seems to be more pronounced in carriers of Apo E4 alleles (Poirier et al., 1993; Payami et al., 1996; Farrer et al., 1997; Bretsky et al., 1999; Breitner et al., 1999; Molero et al., 2001).

Based on these epidemiological studies it was suggested that the lack of protective gonadal hormones, especially estrogen, after menopause may increase the risk for AD in females. Some biochemical studies support this notion as decreased levels of gonadal hormones or elevated sex-hormone binding globulin have been measured in AD patients (Manly et al., 2000; Hoskin et al., 2004), however the issue is controversial (Hogervorst et al., 2003). Consequently, several retrospective studies have established that use of hormone replacement therapy during menopause was associated with a reduced risk for sporadic AD (Henderson et al., 1994; Paganini-Hill and Henderson, 1994; Tang et al., 1996; Baldereschi et al., 1998; Waring et al., 1999; Steffens et al., 1999; Slioter et al., 1999) – although, again, conflicting results have been published (Brenner et al., 1994; Seshadri et al., 2001). Nevertheless, prospective clinical trials have been initiated utilizing gonadal hormones in the treatment of AD, with only marginal (Fillit et al., 1986; Schneider et al., 1996; Yoon et al., 2003) or no benefit at all (Mulnard et al., 2000; Wang et al., 2000). The disappointing results from hormone therapy as a

treatment option for AD have shifted the focus of attention on the possibility that hormones may rather be effective in the *prevention* of AD. But again, despite positive results from initial, smaller studies, the results of recent large clinical trials were disappointing (Henderson et al., 2000; Seshadri et al., 2001; Shumaker et al., 2003).

In conclusion, the overall results from studies utilizing gonadal hormones in the prevention or treatment of AD led to the current recommendation that – due to limited or no efficacy but considerable side effects – hormone replacement therapy can not be recommended, neither for the treatment nor the prevention of AD (Henderson, 2004).

Despite several further questions to be explored, the negative results from hormone studies may allow to conclude that hormone treatment does not have a major impact on the development of AD or may even be harmful, based on results from a recent trial where hormone replacement therapy even increased the risk to suffer from probable dementia (Shumaker et al., 2003). This would be in accordance with our data in Thy1-APP transgenic mice, where a gender-disparate higher formation of A β was detectable in female mice from an age of 3 months and still present in 12 months old female mice, although a considerable proportion of female mice should already be acyclic at that stage. Hence, it can be concluded that female gonadal hormones rather may increase A β formation, which may be related to the above-mentioned higher risk for dementia after hormone replacement therapy. Alternatively, other factors apart from gonadal hormones may determine the gender-disparate formation of A β . In this respect, it was recently demonstrated in cell culture that luteinizing hormone can increase β -amyloidogenic cleavage of APP and that treatment of mice with leuprolide, which suppresses gonadotropin secretion and subsequently the synthesis of gonadal hormones, can reduce formation of A β in female mice *in vivo* (Bowen et al., 2004). Although no comparable findings in humans are available, these results nevertheless suggest that a higher risk for AD in women may be related to increased levels of gonadotropins after menopause, which may negatively affect APP processing and lead to accumulation of A β especially in females (Johnson et al., 1998; Kraszpulski et al., 2001; Corder et al., 2004).

Apart from differences after menopause, it may also be possible that gender differences exist, cumulating over the whole life time, which increase the risk to develop AD. Consequently, the results from Thy1-APP transgenic mice suggest that female mice show an inherent higher β -amyloidogenic cleavage of APP, which seems to be independent of gonadal function as it was still present in 12 months old mice.

Therefore, hormone-independent factors like e.g. differences in brain morphology and neuron number between males and females (Breedlove, 1992; Carrer and Cambiasso, 2002), which are found in rodents (De Vries, 2003) as well as in humans (Pakkenberg et al., 2003), may explain a different sensitivity of female versus male brain tissue towards AD-specific neurodegenerative processes. Further exploration of these gender differences may shed more light on AD-relevant pathogenic processes.

4.4.4 Is it possible to prevent AD by antioxidants?

Several therapeutic approaches for treatment of AD are focusing on the use of antioxidants, but this rationale is mainly based on one clinical trial (Sano et al., 1997) where extremely high doses of vitamin E (2000 IU daily) were able to delay the progression of disease. However, the endpoint in this study was poorly defined including a relatively large variety of events, and the results were only significant after adjustment for the baseline MMSE scores, as the placebo and treatment group differed in this parameter despite randomization. Nevertheless, antioxidants could be explored as further therapeutic options or in the prevention of AD, with the advantage that side-effects are usually either none (vitamin E) or only mild (vitamin C) – with the exception of vitamin A (Beta Carotene Cancer Prevention Study Group, 1994; Albanes et al., 1996; Omenn et al., 1996). However, for a causal therapy it has to be established first whether oxidative stress is a primary event in the disease pathogenesis or just a side-effect of the neurodegenerative process.

Although our results in general confirm that oxidative stress plays a role in the pathogenesis of AD, we can not completely establish whether oxidative damage is indeed causally related to the disease progress. However, some observations argue for oxidative damage as an early event in the pathogenesis. First of all, our data on sporadic AD patients show that HNE levels can be elevated in early stages of the disease, where the clinical degree of cognitive impairment is relatively moderate. This is in line with studies that oxidative markers are quantitatively highest in early stages of the disease (Nunomura et al., 2001; Abe et al., 2002). Also, studies utilizing brain tissue from subjects with mild cognitive impairment, which is considered to be a prodromal stage of AD (Grundman et al., 2004), have found increased markers for oxidative stress (Pratico et al., 2002; Rinaldi et al., 2003; Mecocci, 2004). Furthermore, our results on Thy1-APP transgenic mouse brains revealed that increased HNE levels can be detected as early as at 3 months of age and even before the deposition of plaques, in good accordance with

another study of a different APP transgenic mouse model (Pratico et al., 2001). Hence, it can be speculated that oxidative damage constitutes an early event in the pathogenesis of AD.

In addition, some experimental evidence argues for manifold causal roles of oxidative stress in accelerating A β accumulation, either *in vitro* by increasing amyloid aggregation (Dyrks et al., 1992) or *in vivo*, as it was recently shown that breeding APP transgenic mice on a heterozygous MnSOD knockout background, which causes oxidative stress, increases the accumulation of A β levels and plaque burden in brains from these mice (Li et al., 2004a). Furthermore, early vitamin E supplementation in young but not in aged APP transgenic mice reduces lipid peroxidation markers, A β levels and plaque burden (Sung et al., 2004). Apart from A β toxicity and plaque pathology, oxidative stress might also be involved in the formation of neurofibrillary tangles (Mattson et al., 1997; Perez et al., 2002) and be causally relevant for loss of nicotinic and muscarinic acetylcholine receptors in AD brains (Fawcett et al., 2002; Yu et al., 2003). This evidence suggests that the buildup of both major neuropathological features plaques and tangles as well as loss of acetylcholine receptor function in AD brains may be prevented or slowed down by administration of antioxidants.

Hence, it seems possible that an early intervention by treatment with antioxidants or, even more promising, preventive approaches can slow disease progression or even reduce the risk for development of AD. In accordance with this notion, recent studies reported a reduced risk of AD in subjects with a high intake of antioxidants, where vitamin C and E were proven to be effective either alone (Morris et al., 1998; Engelhart et al., 2002; Morris et al., 2002a) or only in combination (Zandi et al., 2004). Although these studies were prospective, the design was only observational, so that a multitude of antioxidant supplements and various dosage regimens are included. Furthermore, the conclusion that antioxidant use is effective in prevention of AD can not be drawn with certainty from these studies because concentrations of antioxidants were not measured directly in blood samples. Therefore, some speculation remains about the validity of antioxidant intake assessments. Nevertheless, these studies spur further motivation for investigation of this issue in an interventional design. Several of such studies have been initiated, but results will become available only in months ahead. Nevertheless, there is already considerable evidence today that augmentation of antioxidant defenses may delay the onset of AD, which would at least reduce the prevalence of this disease in the future.

5 SUMMARY / ZUSAMMENFASSUNG

5.1 Summary

The aim of this thesis was to investigate a putative contribution of oxidative stress to the pathogenesis of Alzheimer's disease (AD) in several different models. By analyzing various parameters related to oxidative stress in transgenic mouse models based on familial AD (FAD) mutations and in human post-mortem brain tissue from sporadic AD patients, a comprehensive approach should be undertaken to identify causal disease mechanisms triggered by FAD mutations and sporadic AD risk factors. Furthermore, the relevance of transgenic mice compared to studies of brains from sporadic AD patients could be assessed.

5.1.1 The influence of aging studied in mice

In the first part of this thesis, the influence of aging, the most important risk factor for sporadic AD, on oxidative stress-related markers was analyzed in mice. For these studies, brain tissue and splenic lymphocytes as a peripheral cell model from C57BL/6J mice were studied, as the C57BL/6J strain is the genetic background for the FAD mouse models analyzed in further studies in this thesis. In brain tissue from these mice, aging led to an upregulation of the activities of Cu/Zn-superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR), while levels of lipid peroxidation products malondialdehyde (MDA) and 4-hydroxynonenal (HNE) were simultaneously reduced in aged animals. Also *in vitro* stimulation of lipid peroxidation yielded reduced or unchanged MDA levels in aged animals, suggesting that antioxidant enzymes provide a major contribution to the protection against oxidative damage *in vivo* and *in vitro*. In splenic lymphocytes, mitochondrial reactive oxygen species (ROS) levels were only slightly but not significantly increased in aged animals.

Furthermore, profound gender differences were observed in C57BL/6J mice. In brain tissue, female mice showed elevated GPx activity and reduced basal and stimulated levels of lipid peroxidation products compared to male mice. Similarly, splenic lymphocytes from female mice displayed lower levels of mitochondrial ROS. Therefore, female mice are seemingly better protected against ROS accumulation and oxidative damage in brain tissue as well as peripheral cells, and higher GPx activity probably contributes to these observations.

The overall conclusion from the studies on C57BL/6J mice is that physiological aging in healthy animals leads to a compensatory upregulation of antioxidant enzymes, which

play a primary role in preventing excessive ROS accumulation and oxidative damage.

5.1.2 Effects of FAD mutations on oxidative stress parameters

The effects of FAD mutations on oxidative stress parameters were assessed in experiments on transgenic mice bearing mutant human PS1 and/or APP.

In PS1M146L transgenic mice, increased oxidative damage could be detected only in aged 19-22 months old animals, which argues for a higher susceptibility towards oxidative stress caused by the aging process. In brain tissue, HNE levels were 34.8 % increased in PS1M146L compared to PS1wt mice, while MDA levels were not altered. Antioxidant defense was not impaired, as neither were the activities of antioxidant enzymes reduced nor was the *in vitro* stimulation of lipid peroxidation altered in samples from PS1M146L mice. In splenic lymphocytes from PS1M146L mice, which express the transgenic PS1, mitochondrial and cytosolic ROS were elevated to 142.1 % and 120.5 % the levels of non-transgenic mice, respectively. In summary, the PS1M146L mutation exerts oxidative toxicity in aged mice via a mechanism of increased ROS formation. Although severe damage to mitochondria could be excluded from our experiments, the exact steps leading to ROS accumulation remain to be elucidated.

As further AD mouse models, mice bearing PDGF-APP and double transgenic PDGF-APP/PS1 mice were studied. No changes in any of the parameters analyzed could be detected in brains from these mice in any age group. PDGF-APP transgenic mice form only low levels of A β , but double transgenic PDGF-APP/PS1 mice show profound accumulation of A β during aging, as detected by Western blot and ELISA, and have been described to display A β plaques from an age of 6 months. Hence, the mere presence of A β plaques is not sufficient to trigger oxidative stress.

In contrast to PDGF-APP transgenic mice, Thy1-APP transgenic mice show pronounced oxidative damage. These mice express similar levels of full-length APP as PDGF-APP transgenic mice, but show elevated β -secretase cleavage of APP with much higher formation of the C-terminal fragment C99 and A β . Increased levels of HNE could be detected from an age of 3 months (115 % of non-transgenic controls), which was even more pronounced and statistically significant at 12 months of age (184 % of non-transgenic controls). These increased HNE levels were accompanied by reduced activity of Cu/Zn-SOD in 3 and 12 months old animals (93.4 % and 91.2 % of non-

transgenic controls, respectively), suggesting that impaired antioxidant defence by Cu/Zn-SOD is causally responsible for increased formation of HNE. However, no alterations were observed in 18 months old animals, which may be due to adaptive processes – like the upregulation of antioxidant enzymes observed previously in C57BL/6J mice during aging. In the age groups of 3 and 12 months, both male and female transgenic mice were studied, and profound gender differences were observed. Although both gender show similar expression of full-length APP in brain tissue, female Thy1-APP transgenic mice display higher β -secretase cleavage resulting in increased formation of C99 and A β compared to male mice. Both the pools of insoluble as well as soluble A β were approximately 30 % increased in female mice. Therefore, increased formation of C99 and subsequently A β from APP by increased β -amyloidogenic cleavage but not altered aggregation or deposition of A β in brain tissue is responsible for the gender difference in A β levels. Consistent with increased formation of A β and/or C99 in female mice, increased levels of HNE and reduced activity of Cu/Zn-SOD could be detected earlier in female compared to male Thy1-APP transgenic mice, i.e. already at an age of 3 months, suggesting that increased β -amyloidogenic cleavage of APP with increased C99 and/or A β formation causes impaired Cu/Zn-SOD activity and effects increased formation of HNE. Furthermore, as Thy1-APP transgenic mice have been described to display formation of A β plaques from an age of 6 months, oxidative damage constitutes an early event in this mouse model and occurs independently from the presence of plaques, which again confirms our previous observations in PDGF-APP/PS1 mice that A β plaques do not provoke oxidative stress in APP transgenic mice.

In an independent study, reduced activity of Cu/Zn-SOD was also found in another APP transgenic mouse model, APP23 mice. This confirms the observations on Thy1-APP mice, i.e. that overexpression of APP with familial AD mutations can lead to reduced activity of Cu/Zn-SOD. Therefore, our studies identify impaired Cu/Zn-SOD activity as a novel and causal mechanism for oxidative toxicity of APP mutations.

5.1.3 Studies on human brain tissue from sporadic AD patients

In order to analyze the influence of sporadic AD risk factors and for a comparison of the transgenic mouse models based on FAD mutations with the much more common sporadic form of AD, post mortem brain tissue from sporadic AD patients was analyzed in the final part of this thesis.

In a first set of human tissue samples, brain tissue from four different brain regions, i.e. frontal, parietal and temporal cortex as well as cerebellum, was analyzed. The major findings were that activities of antioxidant enzymes were upregulated in AD samples in several brain regions, of which samples from temporal cortex – a brain region that is severely affected by the disease – showed the most pronounced changes. Levels of lipid peroxidation products MDA and HNE were not elevated in AD samples. However, a trend towards lower levels of HNE in cerebellum was found in control and AD brains, suggesting that low levels of this aldehyde may be responsible for the resistance of cerebellar neurons against neurodegeneration in AD. The functional relevance of upregulation of antioxidant enzyme activities was confirmed by *in vitro* stimulation of lipid peroxidation, where significantly lower levels of MDA were formed in samples from AD patients. Furthermore, the observed alterations were modified by gender, as female AD patients showed higher activities of Cu/Zn-SOD and GPx, but not GR, compared to male patients. HNE levels were simultaneously increased, suggesting that brains from female patients are more vulnerable towards oxidative damage – despite the upregulation of Cu/Zn-SOD and GPx.

In a second set of samples, only tissue from temporal cortex was analyzed in a larger number of samples. Again, activities of antioxidant enzymes Cu/Zn-SOD (119.2 % of controls) and GPx (120.9 % of controls), but not GR, were increased in AD samples. This was accompanied by increased levels of HNE (131.8 % of controls), but not MDA. Furthermore, *in vitro* stimulation of MDA formation led to increased levels in AD samples, suggesting that upregulation of Cu/Zn-SOD and GPx in AD brains is not sufficient to protect against oxidative damage.

As A β is hypothesized to be the main toxic factor in AD pathogenesis, levels of soluble A β_{1-40} were determined and shown to be increased in AD brains (415 % of controls), but there was a considerable overlap between AD patients and controls, suggesting that soluble A β_{1-40} can not be the only toxic agent in AD pathogenesis. Nevertheless, A β levels were positively correlated with MDA levels and activities of Cu/Zn-SOD and GPx, and showed a trend towards positive correlation with GR. Hence A β can cause oxidative damage and at the same time induce upregulation of antioxidant enzymes.

Results were furthermore evaluated according to the Apo E4 genotype, which is the strongest genetic risk factor associated with sporadic AD known so far. The Apo E genotype has a major influence on the levels of A β , which were lowest in carriers of

Apo E2 alleles and highest in homozygous Apo E4 carriers. Furthermore, lipid peroxidation products MDA and HNE were modulated by Apo E4 genotype with a gene dosage effect, as levels of both aldehydes were lowest in non-E4 carriers and highest in homozygous Apo E4 carriers, although these differences failed to reach statistical significance due to relatively low numbers of subjects in the respective subgroups. Nevertheless, they confirm that the AD risk factor Apo E4 increases oxidative damage in brain tissue, while the activities of antioxidant enzymes are not affected.

Important information as to putative alterations of oxidative stress-related parameters with the progression of AD could be obtained from correlations with MMSE scores, which are a measure of cognitive abilities. Interestingly, MDA levels were negatively correlated with MMSE scores, indicating that oxidative damage at the level of MDA increases with the clinical progression of disease. HNE levels showed however a different pattern, as they were highest AD patients with moderate cognitive impairment and receded to almost the levels of controls in severely affected AD patients. Hence, MDA and HNE detect different kinds of oxidative damage during progression of AD, and HNE levels may be especially important for initiation of neurodegenerative events early in the disease pathogenesis.

In summary, alterations in oxidative stress-related parameters were found in brains from sporadic AD patients, and these changes were additionally shown to be correlated with A β levels and to be modulated by gender and the Apo E4 genotype. These results support the hypothesis that oxidative stress, being subject to an influence of important risk factors for sporadic AD, contributes to the pathogenesis of Alzheimer's disease in humans.

5.1.4 Comparative summary and conclusions

As a novel approach to investigate the pathogenesis of AD, different models for the disease have been directly compared in the course of this thesis. Taken together, oxidative damage could be detected in brains from sporadic AD patients as well as in various transgenic mouse models, although different causative mechanisms seem to operate in the different models, i.e. increased ROS formation by the PS1M146L mutation, impaired antioxidant defense via Cu/Zn-SOD caused by APP mutations and insufficient compensative upregulation of antioxidant enzymes in sporadic AD patients. Nevertheless, these different mechanisms consistently converge in the accumulation of

oxidative damage, which underscores the importance of these findings. Hence it could be demonstrated that various risk factors for sporadic AD as well as the presence of FAD mutations result in oxidative stress as common final pathway.

Interestingly, in transgenic mouse models as well as in human brain tissue, only levels of HNE but not MDA were elevated, suggesting that the two lipid peroxidation aldehydes detect different kinds of oxidative damage, where HNE is the more specific marker for lipid peroxidation processes in AD-relevant models. Furthermore, as levels of neurotoxic HNE are elevated in sporadic AD patients with relatively moderate cognitive impairment and can be detected as early as in 3 months old Thy1-APP transgenic mice, it seems that HNE can constitute an early and causative factor for neurodegenerative processes in Alzheimer's disease.

Hence, the results of this thesis strongly support the further exploration of pharmacological approaches that augment antioxidant defenses for the prevention and/or treatment of AD.

5.2 Zusammenfassung

Ziel dieser Arbeit war es, einen möglichen Beitrag von oxidativem Stress zur Pathogenese der Alzheimer Krankheit in unterschiedlichen Modellen zu erforschen. Die Untersuchung verschiedener Parameter, die in Zusammenhang mit oxidativem Stress stehen, – sowohl in transgenen Mausmodellen, beruhend auf familiären Alzheimer-Mutationen, als auch in humanem post mortem Gehirngewebe von sporadischen Alzheimerpatienten – sollte eine umfassende Analyse zur Identifizierung kausaler Krankheitsmechanismen ermöglichen, die durch familiäre Alzheimer-Mutationen und durch Risikofaktoren für die sporadische Alzheimer Krankheit ausgelöst werden. Zudem könnte so die Relevanz transgener Mausmodelle im Vergleich zu Studien an Gehirngewebe von sporadischen Alzheimerpatienten beurteilt werden.

5.2.1 Alterseffekte in Mäusen

Im ersten Teil dieser Arbeit wurde der Einfluss des Alterns, was den wichtigsten Risikofaktor für die sporadische Alzheimer Krankheit darstellt, auf oxidativen Stress in Mäusen untersucht. Dafür wurden Gehirngewebe und Milzlymphozyten als peripheres Zellmodell von C57BL/6J Mäusen verwendet, da dieser Mausstamm den genetischen Hintergrund der transgenen Mäuse bildet, die für weitere Untersuchungen im Rahmen dieser Dissertation eingesetzt wurden. Im Gehirngewebe dieser Mäuse führte der Alterungsprozess zu erhöhten Aktivitäten der Enzyme Cu/Zn-Superoxiddismutase (Cu/Zn-SOD), Glutathionperoxidase (GPx) und Glutathionreduktase (GR), während gleichzeitig die Spiegel an Lipidperoxidaionsprodukten Malondialdehyd (MDA) und 4-Hydroxynonenal (HNE) in alten Tieren erniedrigt waren. Auch die *in vitro* Stimulation der Lipidperoxidation führte zu erniedrigten oder unveränderten MDA-Spiegeln in gealterten Tieren, so daß antioxidative Enzyme einen großen Beitrag zum Schutz gegen oxidative Schäden *in vivo* und *in vitro* leisten. In Milzlymphozyten von alten Tieren waren die Spiegel an mitochondrialen reaktiven Sauerstoffspezies (ROS) nur leicht, aber nicht signifikant erhöht.

Außerdem wurden deutliche Geschlechtsunterschiede in C57BL/6J Mäusen beobachtet. Im Gehirngewebe zeigten weibliche Mäuse erhöhte GPx-Aktivität und erniedrigte basale und stimulierte Spiegel an Lipidperoxidaionsprodukten im Vergleich zu männlichen Mäusen. Gleichmaßen wiesen Milzlymphozyten von weiblichen Mäusen niedrigere Spiegel an mitochondrialen ROS auf. Daher sind weibliche Tiere

anscheinend besser gegen die Akkumulation von ROS und oxidative Schäden sowohl im Gehirn als auch in peripheren Zellen geschützt, was wahrscheinlich durch die höhere GPx-Aktivität verursacht wird.

Insgesamt lässt sich also aus den Untersuchungen an C57BL/6J Mäusen schließen, dass der physiologische Alterungsprozess in gesunden Tieren kompensatorisch zu einer Erhöhung der antioxidativen Enzymaktivitäten führt, die eine wichtige Rolle im Schutz gegen Akkumulation von ROS und oxidative Schäden spielen.

5.2.2 Einfluß von familiären Alzheimer-Mutationen

Der Einfluss von familiären Alzheimer-Mutationen auf oxidative Parameter wurde in Experimenten an transgenen Mäusen untersucht, die menschliches PS1 und/oder APP mit Mutationen tragen.

In PS1M146L-transgenen Mäusen wurden erhöhte oxidative Schäden nur in 19-22 Monate alten Tieren gefunden, was dafür spricht dass der Alterungsprozess eine erhöhte Empfindlichkeit gegenüber oxidativem Stress verursacht. Im Gehirngewebe waren die HNE-Spiegel in PS1M146L transgenen Mäusen um 34.8 % im Vergleich zu PS1wt transgenen Mäusen erhöht, wohingegen die MDA-Spiegel unverändert waren. Antioxidative Mechanismen sind dabei nicht beeinträchtigt, da weder die Aktivitäten der antioxidativen Enzyme erniedrigt noch die *in vitro* Stimulation der Lipidperoxidation in Proben von PS1M146L transgenen Mäusen erhöht waren. In Milzlymphozyten, die das transgene PS1 exprimieren, waren mitochondriale und cytosolische ROS jeweils auf 142.1 % und 120.5 % der Spiegel in nicht-transgenen Mäusen erhöht. Zusammenfassend verursacht die PS1M146L Mutation oxidative Schäden in alten Mäusen über einen Mechanismus erhöhter ROS-Bildung. Obwohl eine direkte Schädigung von Mitochondrien in unseren Experimenten ausgeschlossen werden konnte, bleiben die genauen Einzelschritte, die zur Akkumulation von ROS führen, ungeklärt.

Als weitere Mausmodelle wurden PDGF-APP und doppelt transgene PDGF-APP/PS1 Mäuse verwendet. In diesen Mäusen wurden in keiner Altersgruppe Veränderungen in irgendeinem der untersuchten oxidativen Parameter festgestellt. Mittels Western Blot und ELISA konnte gezeigt werden, daß PDGF-APP transgene Mäuse nur sehr niedrige Mengen an A β bilden, wohingegen PDGF-APP/PS1 Mäuse deutliche Akkumulation von A β mit dem Alter zeigen. Zudem wurde die Plaquebildung ab einem Alter von 6

Monaten für diese Mäuse beschrieben. Somit ist die bloße Anwesenheit von A β Plaques nicht ausreichend, um oxidativen Stress auszulösen.

Im Gegensatz dazu zeigen Thy1-APP transgene Mäuse deutliche oxidative Schäden. Diese Mäuse exprimieren ähnliche Mengen an APP wie PDGF-APP transgene Mäuse, weisen allerdings erhöhte β -Sekretasespaltung von APP unter verstärkter Bildung des C-terminalen Fragments C99 und von A β auf. Erhöhte HNE-Spiegel konnten schon im Alter von 3 Monaten gemessen werden (115 % der nicht-transgenen Kontrolltiere), was noch ausgeprägter und statistisch signifikant in 12 Monate alten Tieren war (184 % der nicht-transgenen Kontrolltiere). Diese erhöhten HNE-Spiegel waren begleitet von reduzierter Cu/Zn-SOD-Aktivität in 3 und 12 Monate alten Tieren (jeweils 93.4 % und 91.2 % der nicht-transgenen Kontrolltiere), so daß die beeinträchtigte antioxidative Abwehr durch geringere Cu/Zn-SOD-Aktivität eventuell ursächlich an der erhöhten Bildung von HNE beteiligt ist. Jedoch wurden keinerlei Veränderungen in 18 Monate alten Tieren gesehen, was eventuell auf adaptive Prozesse zurückzuführen ist – wie beispielsweise eine Erhöhung der antioxidativen Enzymaktivitäten mit dem Alter, was in C57BL/6J Mäusen beobachtet wurde. In den 3 und 12 Monate alten Mäusen wurden jeweils männliche und weibliche transgene Mäuse untersucht, und deutliche Geschlechtsunterschiede konnten beobachtet werden. Obwohl die Expression von APP in beiden Geschlechtern gleich ist, zeigen weibliche Tiere erhöhte β -Sekretasespaltung von APP mit verstärkter Bildung von C99 und A β im Vergleich zu männlichen Tieren. Sowohl unlösliches als auch lösliches A β waren in weiblichen Tieren um etwa 30 % erhöht. Deshalb ist eine erhöhte Bildung von C99 und nachfolgend A β durch verstärkte β -Sekretasespaltung von APP, nicht jedoch eine veränderte Aggregation oder Ablagerung von A β im Gehirngewebe für die Geschlechtsunterschiede verantwortlich. In guter Übereinstimmung mit den erhöhten A β -Spiegeln konnten verstärkte HNE-Bildung und erniedrigte Cu/Zn-SOD-Aktivität in weiblichen Tieren früher als in männlichen Tieren, nämlich schon in einem Alter von 3 Monaten, gemessen werden, so daß wahrscheinlich die vermehrte β -Sekretasespaltung von APP mit erhöhten C99- und/oder A β -Spiegeln ursächlich für die verringerte Cu/Zn-SOD-Aktivität und erhöhten HNE-Spiegel verantwortlich ist. Außerdem treten oxidative Schäden zu einem frühen Zeitpunkt in Thy1-APP transgenen Mäusen und unabhängig von der Plaquebildung auf, die in diesem Mausmodell ab einem Alter von 6 Monaten beschrieben ist. Dies bestätigt noch einmal die Beobachtungen an PDGF-APP/PS1 transgenen Mäusen, daß A β

Plaques selbst keine oxidativen Schäden in APP-transgenen Mäusen verursachen.

In einer unabhängigen Untersuchung konnte auch in einem weiteren APP-transgenen Mausmodell, APP23 Mäusen, verringerte Cu/Zn-SOD-Aktivität festgestellt werden. Dies bestätigt die Befunde an Thy1-APP-Mäusen, daß Überexpression von APP mit familiären Alzheimer-Mutationen zu verminderter Cu/Zn-SOD-Aktivität führen kann. Unsere Untersuchungen konnten somit eine reduzierte Cu/Zn-SOD-Aktivität als einen neuen und kausalen Mechanismus für oxidative Toxizität von APP-Mutationen identifizieren.

5.2.3 Untersuchungen an humanem Gehirngewebe von sporadischen Alzheimerpatienten

Um den Einfluss von Risikofaktoren der sporadischen Alzheimer Demenz zu untersuchen und die Vergleichbarkeit der auf familiären Mutationen basierenden transgenen Mausmodelle mit der zahlenmäßig sehr viel bedeutenderen sporadischen Form der Alzheimer Demenz beurteilen zu können, wurde im letzten Teil dieser Arbeit post mortem Gehirngewebe von sporadischen Alzheimerpatienten untersucht.

In einer ersten Testreihe wurden Gewebeproben aus vier verschiedenen Gehirnregionen, dem frontalen, parietalen und temporalen Cortex sowie dem Cerebellum, untersucht. Die Aktivitäten der antioxidativen Enzyme waren in verschiedenen Gehirnregionen von Alzheimerpatienten erhöht, wobei die Veränderungen im temporalen Cortex – einer von der Krankheit stark betroffenen Gehirnregion – am deutlichsten waren. Die Lipidperoxidaionsprodukte MDA und HNE waren in Proben von Alzheimerpatienten nicht erhöht. Allerdings wurde eine Tendenz zu erniedrigten HNE-Spiegeln in Proben aus dem Cerebellum beobachtet, so daß spekuliert werden kann, ob die relativ niedrigen Spiegel dieses Aldehyds möglicherweise für die Resistenz der Neurone im Cerebellum gegenüber degenerativen Prozessen bei der Alzheimer Demenz verantwortlich sind. Die funktionelle Bedeutung der erhöhten antioxidativen Enzymaktivitäten wurde durch *in vitro* Stimulation der Lipidperoxidation bestätigt, wobei in Proben von Alzheimerpatienten weniger MDA gebildet wurde. Außerdem werden die beobachteten Veränderungen durch das Geschlecht beeinflusst, da weibliche Alzheimerpatienten höhere Enzymaktivitäten von Cu/Zn-SOD und GPx aufweisen. Dabei waren die HNE-Spiegel erhöht, was darauf hinweist, dass Gehirngewebe von weiblichen Patienten empfindlicher gegenüber oxidativen Schäden ist – trotz der erhöhten Aktivitäten von

Cu/Zn-SOD und GPx.

In einer zweiten Testreihe wurde nur Gewebe aus dem temporalen Cortex an einer größeren Anzahl von Proben untersucht. Wiederum waren die Enzymaktivitäten von Cu/Zn-SOD (119.2 %) und GPx (120.9 % der Kontrollen), nicht jedoch von GR, in Proben von Alzheimerpatienten erhöht. Begleitend wurden höhere Spiegel von HNE (131.8 % der Kontrollen), nicht jedoch von MDA, gefunden. Außerdem führte die *in vitro* Stimulation der MDA-Bildung zu erhöhten Werten in Proben von Alzheimerpatienten, so daß die erhöhten Enzymaktivitäten von Cu/Zn-SOD und GPx nicht ausreichend gegen oxidative Schädigung schützen.

Da angenommen wird, daß A β der wichtigste toxische Faktor bei der Pathogenese der Alzheimer Krankheit ist, wurden die Spiegel an löslichem A β_{1-40} bestimmt. Diese waren in Proben von Alzheimerpatienten stark erhöht (415 % der Kontrollen), allerdings gab es eine beträchtliche Überschneidung zwischen Alzheimerpatienten und Kontrollen, so daß lösliches A β_{1-40} nicht der einzige toxische Faktor bei der Pathogenese der Erkrankung sein kann. Dennoch waren die A β -Spiegel positiv mit MDA-Spiegeln und Aktivitäten von Cu/Zn-SOD und GPx korreliert, und zeigten auch eine Tendenz zu positiver Korrelation mit GR. Dies weist darauf hin, daß A β oxidative Schäden verursachen und gleichzeitig zu einer erhöhten Aktivität antioxidativer Enzyme führen kann.

Die Ergebnisse wurden außerdem nach dem Apo E4 Genotyp ausgewertet, welches der wichtigste bisher bekannte genetische Risikofaktor für die sporadische Alzheimer Demenz ist. Der Apo E Genotyp hat großen Einfluss auf die A β -Spiegel, die in Trägern des Apo E2 Allels am niedrigsten und in homozygoten Apo E4-Trägern am höchsten waren. Außerdem werden die Lipidperoxidationsprodukte MDA und HNE durch Apo E4 gendosisabhängig moduliert, da die Konzentrationen beider Aldehyde in Proben von nicht-E4-Trägern am niedrigsten und in homozygoten E4-Trägern am höchsten waren, obwohl diese Unterschiede aufgrund der geringen Probenzahlen in den jeweiligen Untergruppen nicht signifikant waren. Dennoch bestätigen diese Ergebnisse, daß der Risikofaktor Apo E4 oxidative Schäden in Gehirngewebe erhöht, ohne die Aktivitäten antioxidativer Enzyme zu beeinflussen.

Wichtige Rückschlüsse im Hinblick auf Veränderungen in oxidativen Parametern während des klinischen Fortschreitens der Erkrankung konnten durch Korrelationen mit den MMSE-Werten, einem Maß für kognitive Fähigkeiten, gezogen werden.

Interessanterweise waren die MDA-Spiegel negativ mit den MMSE-Werten korreliert, was darauf hinweist, daß oxidative Schäden auf Ebene des MDA mit dem klinischen Fortschreiten der Erkrankung zunehmen. HNE-Spiegel waren dagegen am höchsten in Alzheimerpatienten mit relativ moderater kognitiver Beeinträchtigung und niedriger, fast auf dem Niveau der Kontrollen, in schwer dementen Patienten. Daher scheinen MDA und HNE verschiedene Arten von oxidativen Schäden während des Fortschreitens der Erkrankung zu erfassen, und erhöhte HNE-Spiegel spielen eventuell eine wichtige Rolle bei der Neurodegeneration zu einem frühen Zeitpunkt der Alzheimer Krankheit.

Zusammenfassend wurden Veränderungen in oxidativen Parametern in Gehirnen von sporadischen Alzheimerpatienten gefunden, und es konnte zusätzlich gezeigt werden, dass diese Veränderungen mit den A β -Spiegeln korrelieren und durch das Geschlecht und den Apo E4 Genotyp beeinflusst werden. Diese Ergebnisse unterstützen die Hypothese, dass oxidativer Stress, welcher durch wichtige Risikofaktoren für die Alzheimer Krankheit moduliert wird, zur Pathogenese der Erkrankung beim Menschen beiträgt.

5.2.4 Vergleichende Zusammenfassung und Fazit

Als neuartiger Ansatz, einen Beitrag zur Aufklärung der Pathogenese der Alzheimer Demenz zu leisten, wurden im Rahmen dieser Arbeit erstmalig unterschiedliche Modelle der Alzheimer Krankheit vergleichend untersucht. Insgesamt konnten oxidative Schäden sowohl in Gehirngewebe von sporadischen Alzheimerpatienten als auch in verschiedenen transgenen Mausmodellen nachgewiesen werden, obwohl unterschiedliche Mechanismen dafür kausal verantwortlich zu sein scheinen, nämlich erhöhte ROS-Bildung durch die PS1M146L Mutation, beeinträchtigte antioxidative Aktivität des Enzyms Cu/Zn-SOD verursacht durch APP-Mutationen und ungenügende kompensatorische Erhöhung der antioxidativen Enzymaktivitäten in sporadischen Alzheimerpatienten. Dennoch bewirken diese Mechanismen gleichermaßen eine Akkumulation oxidativer Schäden, was die wichtige Bedeutung dieser Befunde unterstreicht. Somit konnte gezeigt werden, dass verschiedene Risikofaktoren für die sporadische Alzheimer Demenz wie auch die Anwesenheit von familiären Alzheimermutationen in oxidativem Stress als gemeinsamer Endstrecke resultieren.

Interessanterweise waren im Gehirngewebe von transgenen Mäusen wie auch von

Alzheimerpatienten nur HNE-, nicht jedoch MDA-Spiegel erhöht, so daß die zwei verschiedenen Lipidperoxidationsprodukte unterschiedliche Arten von oxidativen Schäden detektieren, wobei HNE der spezifischere Marker in relevanten Modellen der Alzheimer Krankheit ist. Außerdem waren die HNE-Spiegel in Alzheimerpatienten mit relativ moderater kognitiver Beeinträchtigung am höchsten, und erhöhte HNE-Spiegel können auch schon ab einem Alter von 3 Monaten in Thy1-APP transgenen Mäusen gemessen werden, so daß HNE möglicherweise einen frühen und für die Neurodegeneration kausalen Faktor bei der Alzheimer Krankheit darstellt.

Daher unterstützen die im Rahmen dieser Doktorarbeit erhobenen Befunde grundsätzlich die weitere Erforschung pharmakologischer Ansätze zur Verbesserung der antioxidativen Abwehr als Therapie oder auch zur Prophylaxe der Alzheimer Krankheit.

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7 ABBREVIATIONS

ABBREVIATIONS

A β	amyloid beta
Abs	photometrical absorption unit
AD	Alzheimer's disease
AGE	advanced glycation end product
AICD	APP intracellular domain
Apo E	apolipoprotein E
APLP	APP-like protein
APP	amyloid precursor protein
ARE	antioxidant response element
ATP	adenosin triphosphate
BSA	bovine serum albumine
°C	degrees Celsius
C.I.	confidence interval
DAF	diaminofluorescein
DAF-2-DA	diaminofluorescein-2-diacetate
DCF	dichlorofluorescein
DCFH-DA	dihydrodichlorofluorescein-diacetate
DHE	dihydroethidium
DHR	dihydrorhodamine 123
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxynucleotidetriphosphate
dT	desoxy-thymidin
DTT	dithiothreitol
ECL	enhanced chemoluminescence
EDTA	ethylenediaminetetraacetate
eNOS	endothelial nitric oxide synthase
FACS	fluorescence activated cell sorting
FAD	familial Alzheimer's disease
FADH	flavine adenine dinucleotide, reduced
FSC	forward scatter
FCS	fetal calf serum
g	gramm
<i>g</i>	centrifugal force unit
GPx	glutathione peroxidase
GR	glutathione reductase
h	hour(s)
HBSS	Hank's balanced salt solution
HMGC _o A	hydroxymethylglutaryl-coenzym-A
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonicacid
HNE	4-hydroxynonenal
iNOS	inducible nitric oxide synthase
IP ₃	inositol-1,4,5-triphosphate

IU	international unit
KPI	Kunitz-type serine protease inhibitor
l	litre
MDA	malondialdehyde
MES	2-(N-morpholino)-ethanesulfonic acid
MFH	mouse fetal hemoglobin
min	minute(s)
ml	millilitre
MMSE	mini mental status examination
NAD	nicotine adenine dinucleotide
NADH	nicotine adenine dinucleotide, reduced
NADPH	nicotine adenine dinucleotide phosphate, reduced
nNOS	neuronal nitric oxide synthase
NOS	nitric oxide synthase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PHF	paired helical filament
PMI	post mortem interval
PMSF	phenylmethylsulfonyl fluoride
PS1	presenilin-1
PS1M146L	mutant human presenilin-1 with the M146L mutation
PS1wt	human wildtype presenilin-1
PSAP	presenilin-associated protein
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene difluoride
R123	rhodamine 123
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	reverse transcriptase
sAPP	soluble APP fragment
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second(s)
SOD	superoxide dismutase
vs	versus

8 INDEX OF FIGURES AND TABLES

8.1 Index of figures

Figure 1.1: Pathways of formation and enzymatic detoxification of ROS.....	8
Figure 1.2: Major pathways in ROS-induced apoptotic signaling.	16
Figure 1.3: Neuropathological hallmarks of Alzheimer's disease: senile plaques (left panel) and neurofibrillary tangles (right panel).....	25
Figure 1.4: Classification of Alzheimer's disease.	27
Figure 1.5: Formation of A β peptides from cleavage of APP	30
Figure 1.6: The amyloid cascade hypothesis of Alzheimer's disease.	32
Figure 1.7: Membrane topology of presenilin 1.	36
Figure 2.1: Optical system of Becton Dickinson FACSCalibur flow cytometer.	64
Figure 2.2: Representative density blots (a, d) and histograms (b,c) of splenic lymphocytes analyzed by flow cytometry.....	66
Figure 2.3: Gating strategy for lymphocytes or lymphocyte subpopulations.....	67
Figure 2.4: APP/A β Western blot of Tris lysates from Thy1-APP transgenic mouse brains.....	72
Figure 2.5: Representative DNA gel electrophoresis for genotyping of transgenic mice.	76
Figure 3.1: Effect of aging on levels of lipid peroxidation products in brains from C57BL/6J mice.	83
Figure 3.2: Activities of antioxidant enzymes in brains from C57BL/6J mice during aging.....	85
Figure 3.3: Stimulation of lipid peroxidation with FeCl ₃ in young mice (a) and differences in during aging (b).	86
Figure 3.4: Levels of ROS in splenic lymphocytes from C57BL/6J mice during aging.....	88
Figure 3.5: Gender differences in levels of lipid peroxidation products in mouse brains during aging.	90
Figure 3.6: Gender differences in activities of antioxidant enzymes in mouse brains during aging.	90
Figure 3.7: Gender differences in formation of lipid peroxidation product MDA after in vitro stimulation with FeCl ₃	91
Figure 3.8: Gender differences in ROS levels in splenic lymphocytes during aging.....	92
Figure 3.9: Lipid peroxidation products in aged transgenic mice.	96
Figure 3.10: Activities of antioxidant enzymes in aged transgenic mice.	97
Figure 3.11: Analysis of transgenic PS1 RNA expression in splenic lymphocytes.	98
Figure 3.12: ROS levels in splenic lymphocytes from middle-aged PS1 transgenic mice.	100
Figure 3.13: Basal levels of ROS in lymphocytes from aged PS1M146L transgenic mice.	100
Figure 3.14: Correlation between ROS levels (DHR oxidation) in CD4-positive lymphocytes and apoptosis levels in lymphocytes (7-AAD staining).	101

Figure 3.15: ROS levels in lymphocytes from PS1M146L transgenic mice after stimulation with hydrogen peroxide or d-ribose.	102
Figure 3.16: Basal levels of various ROS in lymphocytes from PS1wt and PS1M146L transgenic mice.....	104
Figure 3.17: ROS levels in lymphocytes from PS1wt and PS1M146L transgenic mice after serum withdrawal and stimulation with hydrogen peroxide.	105
Figure 3.18: Correlation between DHR oxidation in splenic lymphocytes and HNE levels in brains from non-transgenic littermate control mice and mice transgenic for PS1wt and PS1M146L.	106
Figure 3.19: Levels of lipid peroxidation products in Thy1-APP transgenic mice during aging.	109
Figure 3.20: Activities of antioxidant enzymes in Thy1-APP transgenic mice during aging...	110
Figure 3.21: Stimulated levels of MDA in Thy1-APP transgenic mice during aging.	111
Figure 3.22: Effect of gender on lipid peroxidation parameters in 3 and 12 months old Thy1-APP transgenic mice.	112
Figure 3.23: Effect of gender on Cu/Zn-SOD activity in 3 and 12 months old Thy1-APP transgenic mice.....	113
Figure 3.24: Effect of gender on GPx activity in 3 and 12 months old Thy1-APP transgenic mice.	114
Figure 3.25: Gender differences in stimulated MDA levels in 3 and 12 months old Thy1-APP transgenic mice.....	115
Figure 3.26: Cu/Zn-SOD activity is reduced in brains from APP23 mice.....	116
Figure 3.27: Effect of aging on accumulation of soluble A β in Thy1-APP transgenic mice....	118
Figure 3.28: Levels of A β are higher in Thy1-APP compared to PDGF-APP and PDGF-APP/PS1 transgenic mice (a). The largest pool of A β is present in insoluble fractions (b).	119
Figure 3.29: Expression of APP and insoluble A β levels in SDS-lysates from APP transgenic mouse brains.....	120
Figure 3.30: Gender differences in levels of insoluble A β in Thy1-APP transgenic mice.	121
Figure 3.31: Levels of A β ₁₋₄₀ in PDGF-APP and PDGF-APP/PS1 transgenic mice during aging.	123
Figure 3.32: Accumulation of soluble A β ₁₋₄₀ with age (a) and (b) gender difference in Thy1-APP transgenic mice.	125
Figure 3.33: Levels of basal lipid peroxidation products MDA (a) and HNE (b) in AD brains.	127
Figure 3.34: Increased activities of antioxidant enzymes in different brain regions from AD patients.	128
Figure 3.35: Correlation between Cu/Zn-SOD activity and GPx activity in samples from AD patients taken from frontal cortex.	129
Figure 3.36: Stimulated MDA levels are reduced in AD brains.	130
Figure 3.37: Gender difference in HNE levels among AD patients.....	131
Figure 3.38: Gender differences in antioxidant enzyme activities among AD patients.....	132
Figure 3.39: Basal levels of lipid peroxidation products in AD patients.	133
Figure 3.40: Increased activities of Cu/Zn-SOD and GPx in AD patients.....	134

Figure 3.41: Correlation between HNE levels and Cu/Zn-SOD activity.	135
Figure 3.42: Stimulated levels of MDA in AD patients.	136
Figure 3.43: Levels of soluble A β_{1-40} in AD patients and controls.	138
Figure 3.44: A β_{1-40} levels depend on Apo E4 genotype.	139
Figure 3.45: Correlation between MDA and A β_{1-40} levels in controls and AD patients.	140
Figure 3.46: Correlation between antioxidant enzymes Cu/Zn-SOD (a) or GPx (b) and A β_{1-40} levels in controls and AD patients.	141
Figure 3.47: Levels of lipid peroxidation products MDA and HNE in relation to Apo E4 genotype.	142
Figure 3.48: Activities of antioxidant enzymes in relation to Apo E4 genotype.	142
Figure 3.49: HNE levels in controls and AD patients in relation to Apo E4 genotype.	143
Figure 3.50: Correlation between MDA levels and MMSE score.	144
Figure 3.51: Correlation between HNE levels and MMSE score.	145
Figure 3.52: Activities of antioxidant enzymes in AD patients according to cognitive impairment.	146
Figure 4.1: Summary of gender differences in oxidative stress-related parameters observed in mice.	167
Figure 4.2: Summary of changes in oxidative stress-related parameters during aging observed in mice.	170
Figure 4.3: Alterations in ROS caused by PS1M146L in lymphocytes from PS1 transgenic mice.	179
Figure 4.4: Putative mechanisms of increased ROS formation by PS1M146L in transgenic mice.	184
Figure 4.5: Summary of oxidative stress-related alterations observed in different AD transgenic mouse models and putative mechanisms for oxidative toxicity.	202
Figure 4.6: Summary of effects on oxidative stress-related parameters observed in brain tissue from sporadic AD patients.	225

8.2 Index of tables

Table 1.1: Important ROS, their chemical formula and major sources.	3
Table 2.1: Characterization of brain tissue samples analyzed in cohort #1.	56
Table 2.2: Results of histopathological analysis of brain samples from cohort #2.	58
Table 2.3: Properties and staining parameters of ROS-sensitive dyes used in lymphocyte studies.	68
Table 2.4: Instrument settings for FACS analysis of ROS-sensitive dyes.	71

Table 3.1: Age groups and number of mice used for aging studies.	82
Table 3.2: Age groups and numbers of mice used for studies of gender differences during aging.	89
Table 3.3: Numbers and age of transgenic animals used for studies.	95
Table 3.4: Numbers and gender distribution of Thy1-APP and non-transgenic animals used.	107
Table 3.5: Numbers and gender distribution of different APP transgenic mice used for analysis of APP expression and A β formation.....	117
Table 3.6: Summary of correlations between different antioxidant enzyme activities in different brain regions.....	129
Table 3.7: Summary of correlations between different antioxidant enzyme activities in cohort #2.....	134
Table 3.8: Correlations between different antioxidant enzyme activities and levels of lipid peroxidation products MDA or HNE.	135
Table 3.9: Distribution of samples from non-demented controls and sporadic AD patients classified as “responders” or “non-responders” according to the extent of MDA formation after stimulation with ferric iron.	136
Table 4.1: Overview of studies on lipid peroxidation products during aging in mice.	149
Table 4.2: Overview of studies on antioxidant enzyme activities during aging in mice.....	150
Table 4.3: Overview of studies on in vitro stimulation of lipid peroxidation during aging in mice.	153
Table 4.4: Relative reduction of Cu/Zn-SOD activity in Thy1-APP and APP23 transgenic mouse brains.....	197
Table 4.5: Overview on reports of markers for lipid peroxidative damage in AD brains.....	206
Table 4.6: Overview of reports on antioxidant enzyme activities in AD brains.	208
Table 4.7: Comparison of results from two independent studies on human brain tissue samples.	219
Table 4.8: Relative increases in activities of antioxidant enzymes in AD brain tissue from temporal cortex compared to non-demented controls in cohort #1 and #2.	219
Table 4.9: Comparison of Apo E genotype of AD patients in cohort #1 and #2.	221

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- Schüssel, K., Leutner, S., Cairns, N. J., Czech, C., Pradier, L., Müller, W. E., Eckert, A.: Gender differences in antioxidant defense in Alzheimer's disease. 33rd Annual Meeting Soc. Neuroscience, New Orleans, USA, 2003. *Soc. Neurosci.* 2003; Abstracts 407.5.
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9.3 Oral presentations

- Schüssel, K., Leutner, S., Cairns, N. J., Czech, C., Pradier, L., Müller, W. E., Eckert, A.: Impact of gender on oxidative stress parameters in Alzheimer's disease. Abstracts of the 45th Spring Meeting and the 20th Meeting 9-11 March 2004, Mainz, Germany. *Arch. Pharmacol.*, 369 Suppl. 1, R84.
- Schüssel, K., Eckert, A., Müller, W. E.: Oxidativer Stress im Alter und bei Morbus Alzheimer. Vortrag im Rahmen des Tagungswochenendes des Graduiertenkollegs „Arzneimittelentwicklung und -analytik“ 17./18. January 2003.
- Schüssel, K., Eckert, A., Müller, W. E.: Oxidativer Stress und Alzheimer – Untersuchungen an einem transgenen Mausmodell. Vortrag im Rahmen des Tagungswochenendes des Graduiertenkollegs „Arzneimittelentwicklung und -analytik“ 10./11. January 2004.

10 ACKNOWLEDGMENTS / DANKSAGUNG

Ohne einige wichtige Personen wäre die vorliegende Arbeit nicht in dieser Weise zustandegekommen. Mein herzlicher Dank gilt daher:

Prof. Dr. Walter E. Müller, für die Überlassung des Themas, für das in mich gesetzte Vertrauen sowie für die Möglichkeit, an internationalen Kongressen teilzunehmen. Und dafür, dass er im Sommer 2000 auf meine email-Anfrage nach einer Doktorandenstelle so bereitwillig eingegangen ist.

HD Dr. Anne Eckert danke ich ganz besonders für die sehr gute und nette Betreuung, für Ihr Talent, jegliche Art von Kritik und Verbesserungsvorschläge stets konstruktiv zu vermitteln, für die Fähigkeit auch nach manch ergebnislosen Testwochen noch zu motivieren, für die ermöglichten Kooperationen und für die Betreuung im Rahmen der Weiterbildung zum Fachapotheker für Arzneimittelinformation.

Außerdem danke ich auch herzlich Frau Merbitz, die mir beim Vorstellungsgespräch so nett den Weg zum Institut gezeigt hat und sich damals wahrscheinlich noch nicht vorstellen konnte, was das noch für Folgen haben würde. Ebenso Frau Mucha für Ihre Herzlichkeit und Frau Förster für Hilfestellungen bei organisatorischen Problemen jeglicher Art.

Dann möchte ich Frau Bozyk für die tolle Pflege der Mäuse danken – und die Mäuse selbst werden Ihr es wohl auch danken.

Weiterhin Dr. Gunter Eckert und Dr. Ulrich Moser für die Institutsorganisation inklusive Betriebsausflug ins Kleinwalsertal.

Für die Einarbeitung in das Thema, Hilfestellungen organisatorischer Art und die nette Aufnahme in Frankfurt danke ich ganz besonders Dr. Silke Leutner. Weiterhin danke ich Dr. Bettina Fröstl und Astrid Bonert für Tips beim Western Blot. Prof. D. Steinhilber und Christina Arnold (AK Steinhilber) für die Einweisung und Benutzung der Ultrazentrifuge.

Gleichermassen möchte ich Claudia Jourdan danken für das gute Anlernen in den ersten Wochen, für Ihre Hilfe bei der Genotypisierung der Mäuse und für Ihre offenen Ohren auch bei anderweitigen Problemen.

Ina Henke für Ihr offenes, nettes Wesen, für Tennisstunden, Disputationsfotos, (Mit-)Teilen von Lebenserfahrung und dass sie das ganze Institut immer so schön zusammenhält.

Susanne Horner für ihre lebenswerte und unkomplizierte Art, und natürlich für den unvergessenen Australien-Urlaub und etliche gemeinsame nachfolgende Abende.

Uta Keil für das Miteinander im Labor, geteilte Freud und geteiltes Leid, und für unvergessene Freizeiterlebnisse (Skiurlaube, Weiterbildungen, Kongresse, New York...).

Claudia Frey einerseits für die gute Zusammenarbeit bei den Lymphozytenstudien, andererseits noch viel viel mehr für die Anregung von Freizeitaktivitäten (Mal-seh'n-Kino mit „Claire - Se souvenir de belle choses“, Fahrt mit dem Motettenchor nach St. Petersburg).

Kathrin Schulz für den frischen Wind, den sie ins Labor gebracht hat, für Ihr bereitwilliges Mitteilen von Ergebnissen auf meine neugierigen Nachfragen hin, und für

Ihre Begeisterungsfähigkeit für das Forschen schlechthin mit gemeinsamen theoretischen Gedankenexkursen, die man sonst viel zu selten macht.

Stefan Bodora nicht nur für seinen Beistand bei Computerproblemen jeglicher Art.

Hebbes danke ich für das späte Mittagessen, den Eksiccatoröffner, Spülbürsten, Hilfe bei Computerproblemen, Beschriftung der Kaffeetasse, jegliche Freizeitaktivitäten auch zusammen mit Ede, für physikothoretische Kaffeegespräche und ganz besonders aber einfach auch für ALLES, um den Rahmen dieser Seiten nicht zu sprengen.

Caren Hildebrand danke ich für Ihre unschätzbare Art, manche Dinge mit solchen Kommentaren zu belegen, die sie schließlich einfacher machen, was immer ein Fels in der Brandung war. Und nicht zuletzt dafür, dass sie das Rattern des Wasserbads am Photometer jahrelang ertragen hat.

Dr. Gisela Holoubek für ihren Optimismus, der oft ein Lichtblick war!

Andrea Kastl für die freundliche Führung durchs Institut beim Vorstellungsgespräch, was die Entscheidung nochmals leichter machte, und natürlich für die Organisation diverser Wanderwochenenden.

Allen Kollegen im Labor für die in der Regel sehr gute und gemeinschaftliche Zusammenarbeit, inklusive der Freizeitaktivitäten von Fußball (besonderer Dank an Steffen Leutz), Chase-Läufen, Sommerlauf, Skiurlaube bis Oper, Burgfestspielen und nicht zuletzt den Grillfeiern am Biozentrum.

Stefanie Schäfer für die nette Kooperation, den regen Gedankenaustausch und neue Anregungen.

Dr. Lakshmi Srinivasan Raghavan und Prof. Jonathan Hadgraft dafür, dass sie die Begeisterungsfähigkeit für Forschung in mir geweckt haben.

Dann danke ich ganz besonders auch Barbara Kiepfer, die meine Zeit in Cardiff so nett mitgestaltet hat, was die Freude an der Arbeit im Labor, an kniffligen Fragestellungen und letztlich die Entscheidung zur Promotion im Wesentlichen mitgeprägt hat, und vor allem für die unersetzliche Freundschaft über die Jahre danach.

Ausserdem danke ich meinen Mathematik- bzw. Chemielehrern OStR Weigl und OStR Simbeck, dafür daß sie mein Interesse an Naturwissenschaften mit geprägt haben.

Matthias und Andrea natürlich für das Korrekturlesen, aber auch meiner ganzen Familie und Matthias für die Unterstützung und den Rückhalt, und noch vieles mehr, was hier nicht gesagt zu werden braucht...

Die Anfertigung dieser Dissertation wurde unterstützt durch ein Promotionsstipendium für Naturwissenschaftlerinnen im Rahmen des Hochschul- und Wissenschaftsprogramms HWP vom 01. 02. 2001 bis 31. 12. 2003.

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